ORGAN DAMAGE UNDER HYPOXIC STRESS CONDITION AND THEIR THERAPEUTICS APPROACH

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November 2017

Dedicated
to
My Parents

DECLARATION

I hereby declare that the thesis entitled **"Organ Damage under Hypoxic Stress Condition and their Therapeutics Approach"** submitted by me, for the award of the degree of ***Doctor of Philosophy*** to **Delhi Technological University (Formerly DCE)** is a record of bonafide work carried out by me under the guidance of Prof. Pravir Kumar.

I further declare that the work reported in this thesis has not been submitted and will not be submitted, either in part or in full, for the award of any other degree or diploma in this Institute or any other Institute or University.

Place: New Delhi

Date: **Signature of the Candidate**

CERTIFICATE

This is to certify that the thesis entitled **"Organ Damage under Hypoxic Stress Condition and their Therapeutics Approach"** submitted by **Mr. Niraj Kumar Jha** to **Delhi Technological University (Formerly DCE)**, for the award of the degree of "Doctor of Philosophy" in Biotechnology is a record of bonafide work carried out by him. Niraj Kumar Jha has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to our knowledge has reached requisite standards.

The results contained in this thesis are original and have not been submitted to any other university or institute for the award of any degree or diploma.

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(Niraj Kumar Jha)

ABSTRACT

For the maintenance of cellular homeostasis and energy metabolism, an uninterrupted supply of oxygen (O_2) is routinely required in the brain. However, under impaired O_2 (Hypoxia) or reduced blood flow (ischemia), the tissues are not sufficiently oxygenated, which triggers disruption of cellular homeostasis in the brain. Further, hypoxia resulting in reduced O_2 delivery to brain tissues is supposed to cause neurodegeneration in both *in vivo* and *in vitro* models. Similarly, chronic exposure to hypoxia has also been reportedly involved in defective vessels formation. Such vascular abnormalities lead to impaired blood flow, diminished nutrient supply and entry of restricted infiltrates thereby limiting O_2 availability to the brain and cause neurological disabilities. Amongst many factors responsible for the brain damage under hypoxia, altered expression of hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) have been recently identified as the cause of neuronal cell death. Since there is no disease-modifying therapies have been designed for hypoxia treatment and current medications offer only symptomatic relief with a broad range of side effects. Therefore, the need of the hour is to unravel new and safer therapies which can improve disease symptoms against hypoxic insults. Owing to the limitations of sample availability of hypoxic patients and animal model, the current research scenario is revolved around cellular hypoxic models which are an excellent source of large drug screening and easy to maintain. Prior to *in vitro* cellular study, we have done *in silico* analysis of two major proteins including, HIF-1α and VEGF, which are reportedly involved in hypoxia-mediated cellular damage. Consequently, we have targeted these proteins with selected biomolecules in order to regulate their altered expression in the cells. Further, to validate our results we have performed *in vitro* experiment as well, where we used Cobalt(II) chloride ($CoCl_2$) as a hypoxia mimic compound to induce chemical hypoxia in SH-SY5Y and HEK-293 cell line for exploring molecular mechanistic of hypoxia-induced cellular loss and screening of biomolecules in an attempt to curtail disease symptoms.

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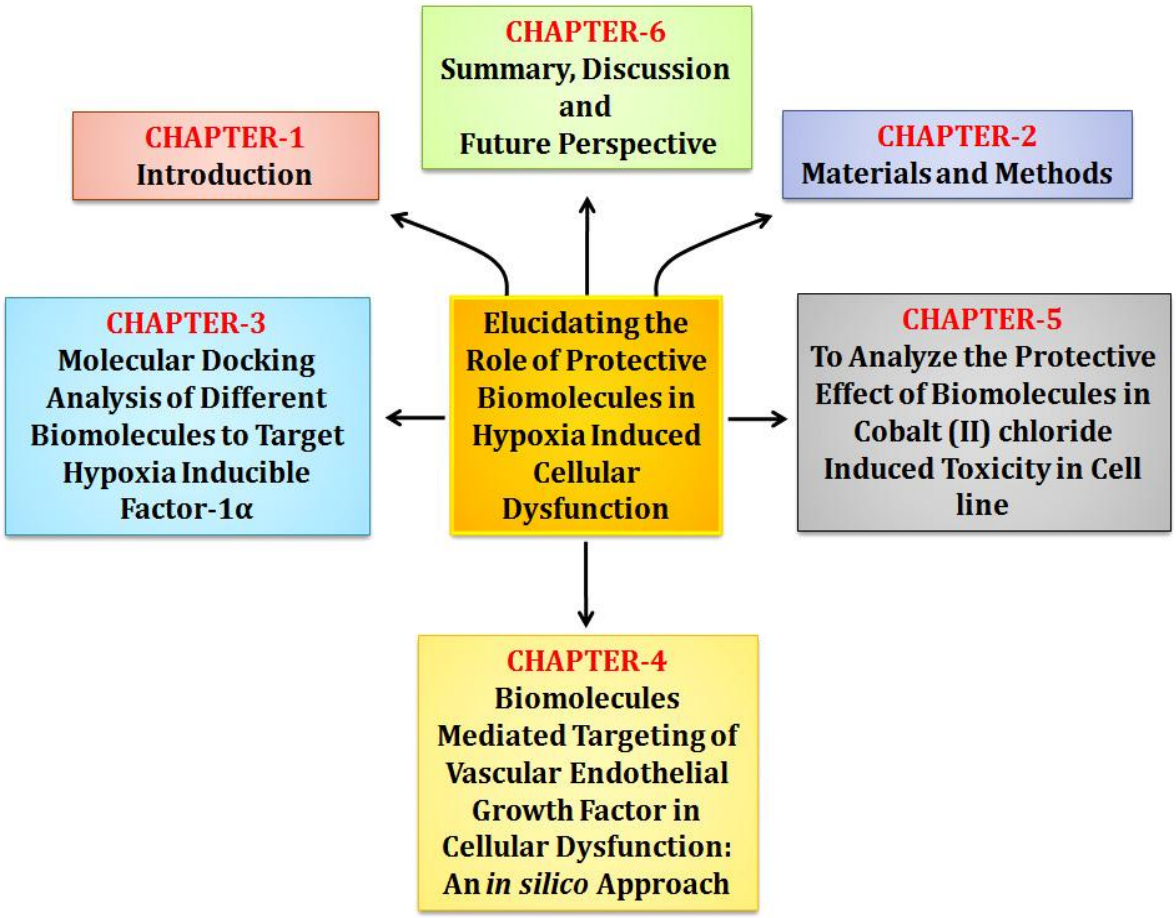


LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
AβPP	Amyloid-β precursor protein
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
CBF	Cerebral blood flow
CIH	Chronic-intermittent hypoxia
CKD	Chronic kidney disease
CNS	Central nervous system
$CoCl_2$	Cobalt (II) chloride
COX	Cytochrome oxidase
CSF	Cerebrospinal fluid
EPO	Erythropoietin
ETS	Electron-transport chain
HACE	High-altitude cerebral edema
HAPE	High altitude pulmonary edema
HD	Huntington's disease
HIF	Hypoxia inducible factor
IL-6	Interleukin 6
LDH A	Lactate dehydrogenase A
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NSCs	Neural stem cells
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PDK1	Pyruvate dehydrogenase kinase 1
PHD	Prolyl-4-hydorxylase
PKC	Protein kinase C
RBCs	Red blood cells

TCA	Tricarboxylic acid cycle
TGF	Transforming growth factor
TNF	Tumour necrosis factor
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau

Chapter I

Introduction and Review of Literature

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 HYPOXIA: AN OVERVIEW

The structural and functional integrity of the cell exquisitely depends on a regular supply of oxygen (O_2). In order to evade the probable damaging outcomes of deficient O_2 availability, the brain triggers endogenous adaptive and pro-survival mechanisms - a phenomenon known as brain hypoxic tolerance. The highly conserved HIF (hypoxia induciable factor) family of proteins and their associated downstream signaling are responsible for these crucial processes (Sharp and Bernaudin 2004; Koh and Powis 2012). Incidentally, any aberrancy in hypoxic signaling results in impaired tricarboxylic acid cycle (TCA cycle) flux, which contributes to diminished energy generation from both the electron-transport chain (ETC) and the citric acid cycle (CAC) thereby leading to overproduction of free radicals (Vatrinet *et al.,* 2017). Further, hypoxia causes disruption in the normal functioning of several crucial processes, including ubiquitin-proteasome system (UPS), mitochondrial dynamics, autophagy, calcium (Ca^{2+}) and iron homeostasis (Ikeda *et al.,* 2014). Impairment of these signaling pathways has pathological implications in the synaptic loss, gradual muscle weakness, loss of motor activity, speech deficit and paralysis, which eventually leads to neuronal death (**Figure 1.1**). Recent evidence has indicated that blood-brain barrier (BBB) dysfunction is one of the major factors for the accumulation of several vasculotoxic and neurotoxic molecules within the brain parenchyma which in turn make neuronal cells prone to hypoxic injury and neuronal degeneration. Importantly, both hypoxia and hypoperfusion have been reported to cause hippocampal degeneration, gray matter (GM) atrophy and progressive cognitive deficits, which lead to neuronal dysfunction (Zlokovic 2011). Furthermore, medical conditions such as asthma, cardiac arrest, pneumonia and stroke cause the body to be deprived of adequate O_2 or a condition called tissue hypoxia. Moreover, extensive athletic training, hiking at high altitudes and diving underwater can lead to hypoxic

condition in the body. In turn, hypoxia can be defined as the insufficiency in the bioavailability of O_2 to the tissues of the body. Further, hiking to high-altitude also causes hypobaric hypoxia and considered as an acute physiological stress. This condition further devlops numerous high-altitude illnesses, including, high-altitude cerebral edema (HACE), high altitude pulmonary edema (HAPE) and hypoxic muscle weakness (Sarkar *et al.,* 2012). Connett and colleagues describe in theory about the three thresholds of cellular hypoxia (Connett *et al.,* 1990).

(1) The first threshold is when cellular O_2 curtails but adenosine triphosphate (ATP) generation is sustained at the level enough to match ATP demand by metabolic adaptation.

(2) The second occurs when steady state ATP yield can be sustained only by the ATP production from anaerobic glycolysis by the Embden-Meyerhof (EMP) pathway. This pathway generates only 2 molecules of ATP per 1 molecule of glucose metabolized. For highly metabolic tissues such as the brain, kidney and liver anaerobic glycolysis is too inefficient to be effective and these organs develop ATP depletion rapidly under hypoxic insults.

(3) The last threshold is when glycolysis gets inadequate to generate sufficient ATP to maintain cell function and structural integrity. Interestingly, most of the tissues after the second threshold build up ATP depletion. One of these tissues is the brain; nerve cells in the brain consume high amount of ATP to activate the sodium-potassium1pump (Na+ /K+ -ATPase). When 50-60% of ATP is lost due to the diminishing levels of O_2, depolarization and uptake of Na+ and H_2O in membrane occurs (Snell *et al.,* 2014). Depolarization results in an influx of Ca^{2+} through voltage-gated Ca^{2+} channels, and leads to glutamate neurotransmitter release. Glutamate binds to glutamate specific receptors and initiating a process of Ca^{2+} influx, thereby causing the death of neurons. Conversely, numerous studies have revealed that some neuronal cells under hypoxic conditions do survive. These surviving cells build up adaptive responses and express elevated levels of HIF-1α, which is a major controller of the cellular response to hypoxia (Rankin *et al.,* 2009; Wenger *et al.,* 2005).

Amongst many factors responsible for the brain damage under hypoxia, altered HIF-1α and VEGF levels have been recently identified as the cause of neuronal cell death. The elevated expression of VEGF in alzhemier's disease (AD) under hypoxia causes abnormal vessel branching, increased vessel diameter, irregular basement membrane and an increased endothelial cell proliferation. Such vascular abnormalities may lead to impaired blood flow, diminished nutrient supply and access of restricted infiltrates (Iyer *et al.,* 1998). Moreover, angiogenesis triggers the expression of various pro-angiogenic markers, including, VEGF, transforming growth factor (TGF) and tumour necrosis factor (TNF) (Greer *et al.,* 2012). The angiogenic activation of all these factors under hypoxic insult contribute to the agglomeration of toxic Aβ and release of a neurotoxic protein that usually hampers cortical neuronal activity and results in the pathophysiology of AD (Semenza 2002). The activity of VEGF protein is triggered under hypoxia through HIF-1α and reported to be upraised in the frontal and para-hippocampal cortex in AD brain (Zhang *et al.,* 2011). While in parkinson's disease (PD), mutations in DJ-1 promoted VHL mediated degradation of the α-subunit (HIF-1α) which further leads to MPP+-induced toxicity in neurons (Ke and Costa 2006). The response to hypoxia involves two crucial cascades, in particular: the HIF-1α pathway and the nuclear factor NF-κB pathway (responsible for the generation of inflammatory mediators). Any alterations in these pathways contribute to the fatal complication of amyotrophic lateral sclerosis (ALS). Defects in VEGF gene expression and deregulation of the HIF-1α pathway are markedly impaired in ALS patients. In case of diabetic nephropathy patients, the VEGF levels were found to be increased and blocking VEGF or VEGF receptor ameliorated the disease symptom of diabetic nephropathy in animal model. Upraised plasma VEGF expressions have also been observed in chronic kidney disease (CKD) patients. Additionally, lethal effects of VEGF have been reported in sepsis and atherosclerosis, which are common hurdle in CKD (Doi *et al.,* 2010).

The successive sections address the holistic overview of hypoxic signaling in the brain. Moreover, we discuss the factors responsible for inducing hypoxic burden in

neurons associated with altered signaling cascades. The pathophysiology of neuronal dysfunction and the involvement of angiogenic/pro-angiogenic and cell proliferation markers to reveal their interlink between hypoxia and neuronal damage have been elaborated. Finally, various therapeutic strategies to target hypoxia along with emerging therapies have been highlighted.

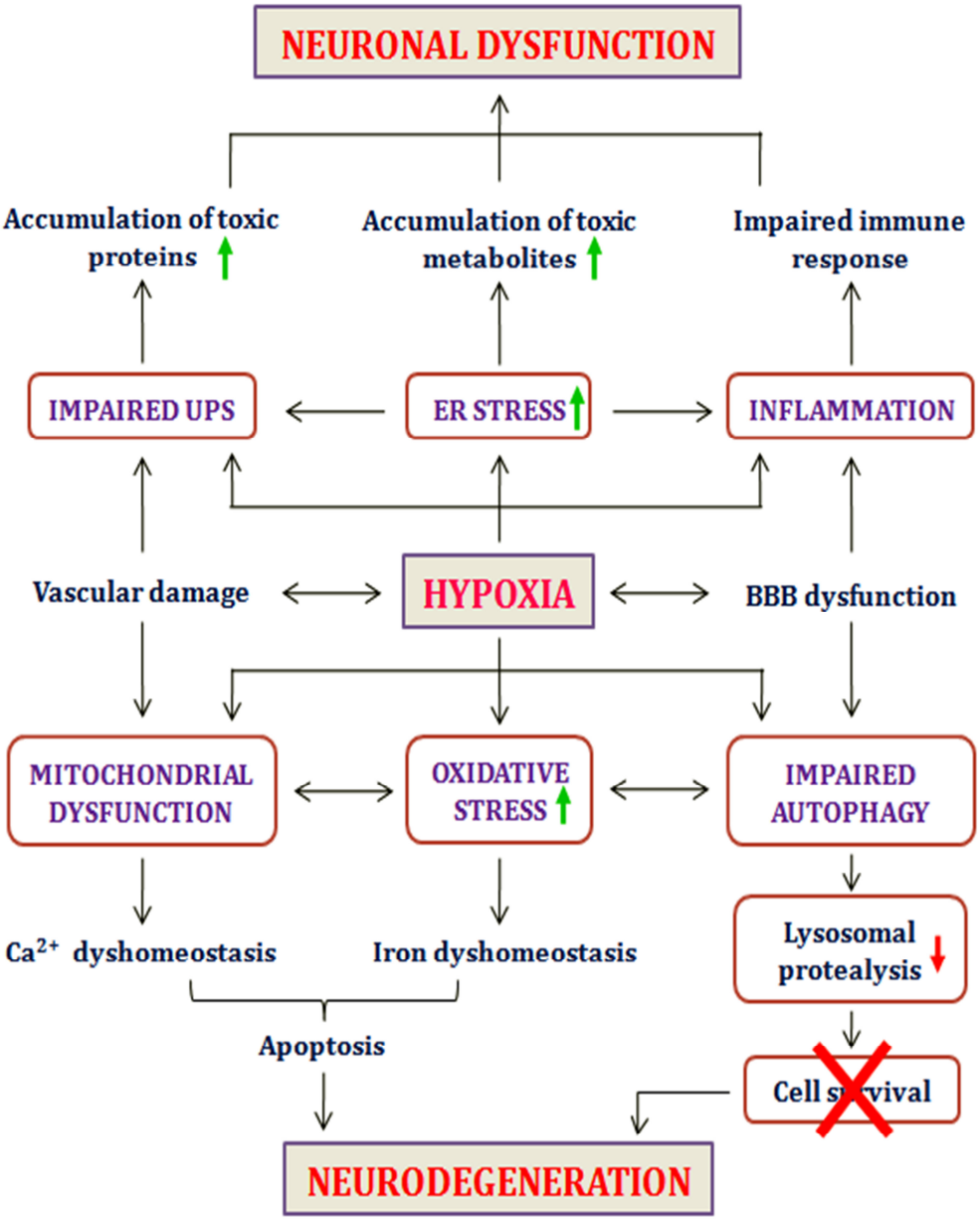


Figure 1.1: Schematic depiction showing the pathological role of hypoxia in neuronal dysfunction

1.2 TYPES OF HYPOXIA

Table 1.1: Types of hypoxia their features and typical causes

Division	Types of Hypoxia	Features	Typical Causes
Oxygen Supply	Hypoxic hypoxia (Hypotonic hypoxia)	Low arterial PO_2	Ventilation-perfusion (V/Q) mismatch, decreased lung diffusion capacity, alveolar hypoventilation, high altitude and intrapulmonary shunt
	Anemic hypoxia (Hemic/Heamogenous)	Decreased total amount of O_2 bound to hemoglobin	Carbon monooxide poisoning, blood loss and anemia (low [HB] or altered HbO_2 binding)
Oxygen Usage	Ishemic hypoxia (Circulatory)	Reduced blood flow	Thrombosis (hypoxia in a single organ), shock (peripheral hypoxia) and heart failure (whole body hypoxia)
	Histotoxic hypoxia (Disoxidative/Histogenous)	Failure of cells to use O_2 because cells have been poisoned	Cyanide and other metabolic poisons

1.3 HYPOXIA MIMICKING COMPOUNDS

To generate hypoxic stress, cells are basically incubated in a hypoxic chamber or treated with chemical compounds that stimulate hypoxic mimic conditions. Owing to the limitations of animal facilities and sample availability of hypoxic patients we induced hypoxic stress in the cell lines by using various chemical inducers. These chemical inducers consist of cobalt (II) chloride ($CoCl_2$), iodochlorohydroxyquinoline, ophenanthroline and desferrioxamine (DFO). $CoCl_2$ has been commonly used in hypoxia related research as it activates hypoxic mimic conditions in cells by inducing HIF-1α expression. However, DFO is also used for mimicking hypoxic condition. It is an iron chelator that chelates the iron in the prolyl-4-hydorxylase (PHD) region of HIF-lα. By decreasing the accessibility of iron, HIF-lα is stabilized because the PHD requires O_2, iron and ascoarbate as cofactors; hence, DFO can trigger downstream signaling as mimicking hypoxic condition. In animal models, DFO induced hypoxia-like adaptive changes in the host. DFO has been shown to increase HIF-lα expression in neuronal cell culture as well (Wu and Yotnda 2011).

1.4 THE MACHINERY OF HYPOXIA: BACKGROUND

Hypoxia is a state of low oxygen tension in the tissues manifested as a result of inadequate oxygen level in blood. The response to hypoxia is orchestrated through activation of the HIF family of transcription factors. The highly conserved HIF family is rightly called the 'headquarter' of homeostatic response of the brain to hypoxia (Kietzmann *et al.,* 2016). Hypoxia-inducible factor 1 **(**HIF-1) consists of an oxygen-sensitive HIF-1 α subunit that heterodimerizes with the Hypoxia-inducible factor 1-beta (HIF-1 β) subunit to bind DNA (Vadlapatla *et al.,* 2013). Under high O_2 tension, HIF-1 α is hydroxylated by prolylydroxylases (PHDs) enzymes. The hydroxylated HIF-1 α subunit thereafter interacts with the von Hippel–Lindau (VHL) protein, an E3 ubiquitin ligase responsible for HIF-1α polyubiquitylation. The ubiquitination maintains the degradation versus synthesis dynamics of HIF-1α under normoxic conditions (Snell *et al.,* 2014). Conversely, HIF-1 α hydroxylation does not occur following hypoxic exposure, thereby stabilizing HIF-1α. Thus, hypoxia induced active HIF-1 complex binds to the core hypoxia response element thereby, triggering an array of diverse processes ranging from energy metabolism, angiogenesis, erythropoiesis, neurogenesis to mitochondrial trafficking and autophagy (**Figure 1.2**) (Wenger *et al.,* 2005). The mechanistic interplay of hypoxia in regulating these processes is comprehensively discussed in succeeding section.

1.4.1 Intricacy of hypoxia in energy metabolism

The best-characterized process regulated by HIF under hypoxia is the energy metabolism in the brain. Under O_2 deprivation in central nervous system (CNS), HIF promotes a metabolic shift from mitochondrial metabolism such as TCA cycle, oxidative phosphorylation and fatty acid oxidation towards glycolysis. This shift from aerobic to anaerobic metabolism is an adaptation of cells to hypoxic stress through generation of ATP (Iyer *et al.,* 1998; Rankin *et al.,* 2009; Solaini *et al.,* 2010). This oxygen dynamics results into numerous crucial phenomena in the neuronal cells. For instance, (1) it causes increased glucose uptake and the subsequent metabolization by

glycolytic enzymes through upregulated expression of glucose transporters (GLUT-1 and GLUT-3), (2) Secondly, HIF-dependent increase in lactate dehydrogenase A (LDH A) converts pyruvate into lactate, (3) reduced pyruvate flow into the TCA cycle leads to HIF-mediated pyruvate dehydrogenase kinase 1 (PDK1) and (4) finally it alternates from cytochrome oxidase (COX) subunit 4 isoform 1 (COX4-1) to the high-efficiency isoform 2 (COX4-2) to promote COX activity under hypoxic insults (Denko 2008; Semenza 2002; Greer *et al.,* 2012). Interestingly, hypoxia is also involved in the pathway that shunts pyruvate and glutamate to alanine and α-ketoglutarate, permitting efficient energy production (Zhang *et al.,* 2011). Moreover, hypoxia induced HIF-1 also increases the production of nicotinamide adenine dinucleotide phosphate (NADPH) for cell survival by regulating glycolysis and hexose monophosphate shunt (HMP shunt) pathway (Mimura and Furuya 1995). Finally, in order to maintain the normal level of hepatic ATP, hypoxia has been found to depress energy expenditure, which is pivotal for cell survival (Zhang *et al.,* 2011).

1.4.2 Hypoxia to new life: Angiogenesis and Erythropoiesis

The multistep process of angiogenesis which is accountable for the formation of new blood vessels from pre existing one is tightly regulated in response to hypoxia. HIF-1 plays a potential role in regulation of hypoxic induction of VEGF. The VEGF recruits endothelial cells to hypoxic

avascular areas in both neural cells and astrocytes and stimulates their proliferation to ameliorate local blood flow and oxygen delivery thereby maintaining the normal brain functioning (Taie *et al.,* 2009; Tang *et al.,* 2013; Ke and Costa 2006). Further, the physiological roles of VEGF in the neural cells are not restricted to angiogenesis. Evidence from the literature reported that VEGF also possesses neurotrophic, neuroprotective and neurogenic properties (Storkebaum *et al.,* 2004; Jin *et al.,* 2002). Apart from driving angiogenesis, hypoxia also contributes to brain metabolism through promoting the generation of new red blood cells (RBCs) to increase the blood O_2-carrying capacity via erythropoiesis (Fan *et al.,* 2009; Noguchi *et al.,* 2007). The

erythropoietin-receptor (EPO-R) is broadly expressed in astrocytes, microglia and endothelial cells of the brain (Nagai *et al.,* 2001). Under hypoxic conditions, erythropoietin (EPO), a gene involved in erythropoiesis activates nitric oxide (NO) to control blood flow and mediated the neurovascular response to hypoxia (Alnaeeli *et al.,* 2012; Wang *et al.,* 2004; Li *et al.,* 2007). EPO is mostly produced by the kidney and the liver cell in an oxygen-dependent manner; while, hypoxic up-regulation of EPO also occurs in numerous brain areas, including the hippocampus, amygdale and temporal cortex (Marti *et al.,* 1996; Digicaylioglu *et al.,* 1995). Hypoxic regulation of EPO was earlier supposed to be controlled by HIF-1, but recent breakthroughs reported that brain EPO transcription is regulated mainly by hypoxia-inducible factor 2 (HIF-2) which in turn directed neurovascular protection and restored local cerebral blood flow (CBF) in a mouse model of focal ischemia (Yeo *et al.,* 2008).

1.4.3 Intimacy of hypoxia and mitochondria: Trafficking and Autophagy

A plethora of evidence has documented the pivotal role of hypoxia in both mitochondrial trafficking and autophagy. In order to ensure proper synaptic function and neurite plasticity, neurons rely on a dynamic axonal transport of mitochondria. Hypoxia has been found to be involved in both anterograde and retrograde transportation of functional and dysfunctional mitochondria to the presynaptic terminals along with microtubules by kinesin and dynein respectively (Li *et al.,* 2004; Sheng and Cai 2012; Chang *et al.,* 2006). This transportation is mainly controlled by Miro and Milton, two outer mitochondrial membrane proteins that bind mitochondria to these motor proteins. Further, a redox-dependent protein known as hypoxia up-regulated mitochondrial movement regulator (HUMMR) has currently been shown to interact with this Miro-Milton complex. In normoxic neurons, HUMMR protein is significantly induced by HIF-1 protein. However, under hypoxic conditions, loss of HUMMR function has been observed to diminish the percentage of anterograde transportation and increase retrograde transportation. Thus, by uplifting the expression of HUMMR, HIF-1 controls mitochondrial trafficking and subsequently leads to the maintenance of neuronal viability and function during hypoxia (Li and

Rempe 2010; Li *et al.,* 2009; MacAskill and Kittler 2010; Wang and Schwarz 2009). Further the cardinal role of hypoxia in mitochondria is not limited to trafficking; it also induces autophagy via induction of HIF. Growing evidences have clearly demonstrated that the hypoxic microenvironment assists to cell survival rather than cell death by activating autophagy (Bellot *et al.,* 2009). Autophagy is an integral piece of the adaptive and pro-survival response of the brain to hypoxia (Balduini *et al.,* 2009). It facilitates the effective clearance of ubiquitinated or aggregated proteins and prevents the agglomeration of toxic aggregates. Interestingly, neurons are extremely dependent on autophagy because of their polarized morphology, post-mitotic nature and active protein trafficking (Lee 2009; Xilouri and Stefanis 2010). It has been reported that Hypoxia-promoted autophagy is mediated through HIF induction of BCL2 Interacting Protein 3 (BNIP3) via their Bcl-2 homology-3 (BH3) domains (Bellot *et al.,* 2009). Further, constitutive expression of Beclin1 (a major actor of autophagy) and Atg5 (E3 ubiquitin ligase) induced via HIF has also been shown as an adaptive metabolic response to hypoxia through selective degradation of mitochondria by autophagy (mitophagy) (Zhang *et al.,* 2008). Thus, hypoxia is intimately related in regulating key functions of mitochondria such as trafficking and autophagy.

1.4.4 Hypoxia and neurogenesis: Friends or Foe?

The term neurogenesis refers to the growth and development of neurons. New functional neurons are routinely generated from neural stem cells (NSCs) throughout life, leading to neuronal plasticity and homeostatic processes in the brain (Bonaguidi *et al.,* 2012). The regions of an adult brain instrumental in neurogenesis are the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone (SVZ), located along the sides of the lateral ventricles (Altman 1962; Eriksson *et al.,* 1998). Evidence has pointed out that the process of neurogenesis is crucial to functions such as learning and memory. Interestingly, hypoxia was shown to trigger neurogenesis in the adult brain (Ohira 2011). The growing evidence reported that the exposure of neural progenitor cells (NPCs) population to reduced oxygen levels augments their proliferation, survival and neuronal differentiation (Studer *et al.,* 2000).

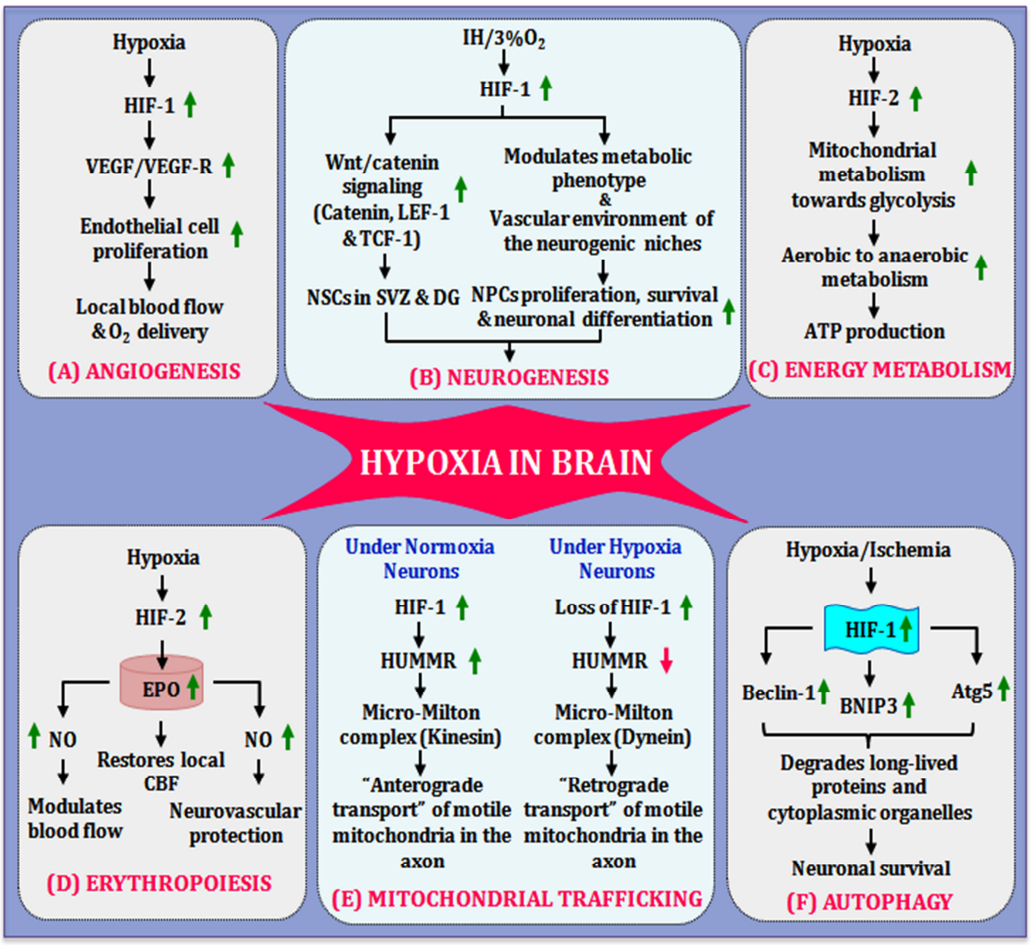


Figure 1.2: The intricate dynamics of Hypoxia in brain. Hypoxia plays numerous crucial roles in cellular metabolism and brain homeostasis through activation of different metabolic pathways. (A) It promotes angiogenesis via endothelial cell proliferation, which is induced by VEGF. (B) The process of neurogenesis is triggered under hypoxic state by the activation of Wnt/catenin signaling. Additionally, (C) the process of energy metabolism is also activated under hypoxic state by dint of promoting aerobic metabolism towards anaerobic metabolism thus lead to the ATP production. Apart from driving angiogenesis, (D) hypoxia also contributes towards brain metabolism through promoting the generation of new red blood cells (RBCs) to increase the blood oxygen-carrying capacity via induction of EPO protein. (E) In case of mitochondrial trafficking, hypoxia acts as a central player for both anterograde and retrograde transportation of mitochondria where, Micro-Milton complex along with motor proteins plays a cardinal role. Finally, (F) the process of autophagy takes place under hypoxia where constitutive expression of both BNIP3 and Atg5 along with Beclin-1 directs degradation of long-lived proteins and cytoplasmic organelles thus lead to neuronal survival. HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor-receptor; IH, intermittent hypoxia; LEF-1, lymphoid enhancer-binding factor-1; TCF-1, T-cell factor-1; NSCs, neural stem cells; SVZ, subgranular zone; DG, dentate gyrus; NPCs, neural progenitor cells; HIF-2, hypoxia-inducible factor-2; EPO, erythropoietin; NO, nitric oxide; CBF, cerebral blood flow; BNIP3, BCL2 Interacting Protein 3; Atg5, autophagy protein 5; HUMMR, hypoxia up-regulated mitochondrial movement regulator

Further, both acute and chronic-intermittent hypoxia (CIH) was found to facilitate the proliferation of NSCs in SVZ and DG. Thus, hypoxic insult supposedly activated endogenous dormant NSCs, which in turn led to proliferation and differentiation of neural progenitors into mature neurons. The mechanism associated with neurogenesis is triggered by the action of HIF-1 under hypoxia. Indeed, both sustained and intermittent hypoxia (IH) is mainly accountable for the activation of HIF-1 in the neurogenic niches (Ross *et al.,* 2012; Cunningham *et al.,* 2012; Nanduri *et al.,* 2008). Further, the molecular mechanisms underlying neurogenesis depicted that HIF-1 regulates Wnt/-catenin signaling in NSCs by increasing catenin activation and expression of the downstream effectors lymphoid enhancer-binding factor-1 (LEF-1) and T-cell factor-1 (TCF-1). On the contrary, HIF-1 deletion altered hippocampal Wnt-dependent processes such as NSCs proliferation, differentiation and neuronal maturation in the SGZ. Besides, numerous findings stated that HIF-1 participated in stem cell differentiation through regulation of Notch and Wnt/-catenin signaling pathways (Gustafsson *et al.,* 2005; Kaidi *et al.,* 2007; Mazumdar *et al.,* 2010). Importantly, HIF-1 also plays a decisive role in neurogenesis by modulating the metabolic phenotype and vascular environment of the neurogenic niches (Cunningham *et al.,* 2012). However, cumulating evidences have pointed out hypoxic stress as an antagonist of neurogenesis. Such insult associated with hypoxia against neurogenesis has been implicated in the development and progression of various neurodegenerative disorders (NDDs).

1.5 PATHOLOGICAL ROLE OF HYPOXIC INJURIES IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is marked by a progressive and gradual decline in cognitive function and memory loss. The major cause of sporadic late-onset form of AD is largely unknown, whereas, familial early-onset AD is typically coupled to mutations in amyloid-β precursor protein (AβPP) and presenilin 1 (PS1) genes (Tarawneh and Holtzman 2012). Mounting evidence has suggested significant involvement of environmental risk factors in the onset and development of AD. Hypoxia is one such

risk factor, which may evoke and exacerbate the disease progression in AD. Further, patients suffering from cerebral ischemia and stroke in which hypoxic milieu occurs have shown high propensity to AD (Peers *et al.,* 2007). Moreover, it is also well-known that hypoxia tilts an aging brain towards AD. Mounting evidence straightens that hypoxia expedites AD progression through amyloid beta (Aβ) agglomeration, tau hyperphosphorylation, BBB dysfunction, impaired calcium homeostasis and advancing the degeneration process of neurons (Zlokovic 2011). Since, periods of chronic hypoxia have been implicated in the clinical and neuropathological course of AD, this section comprehensively explores the contribution of hypoxia in the onset and development of AD.

1.5.1 Pathological angiogenesis: A bridge between hypoxia and Alzheimer's disease

Angiogenesis is one of the major contributors among the various physiological processes known to cause AD. Angiogenesis is an outcome of vascular damage (inflammation) and altered cerebral perfusion (oligaemia) (Zlokovic 2011). The elevated expression of VEGF in AD under hypoxia causes abnormal vessel branching, increased vessel diameter, irregular basement membrane and an increased endothelial cell proliferation. Further, the chronic hypoxia may result in defective vessels formation; i.e. pathological angiogenesis. Such vascular abnormalities may lead to impaired blood flow, diminished nutrient supply and entry of restricted infiltrates (Desai *et al.,* 2009). Moreover, angiogenesis triggers the expression of various pro-angiogenic markers, including TGF, VEGF and TNF (Ucuzian *et al.,* 2010). The angiogenic activation of all these factors under hypoxic insult leads to agglomeration of Aβ peptides and secretion of a neurotoxic peptide that usually hampers cortical neuronal activity and results in pathophysiology of AD (Vagnucci AH and Li 2003). In case of AD, elevated levels of transforming growth factor beta (TGFβ) and VEGF were reported in the cerebrospinal fluid (CSF) that signifies the possible involvement of angiogenesis in its pathogenesis (Tarkowski *et al.,* 2002). On the contrary, Aβ plaques

and neurofibrillary tangles (NFTs), the pathological culprit of AD brains, may also act as an angiogenic marker under hypoxia (Braak and Braak 2007). The Aβ peptides were shown to direct pro-angiogenic as well as anti-angiogenic activity *in vitro* (Paris *et al.,* 2004; Cantara *et al.,* 2004; Boscolo *et al.,* 2007). Moreover, VEGF co-localized with Aβ peptides in response to hypoxia stress (Yang *et al.,* 2004). Interestingly, accumulation of Aβ and hypoperfusion are likely to cause the upregulation of VEGF gene. The activity of VEGF protein is triggered under hypoxia through HIF-1α and found to be upraised in the frontal and para hippocampal cortex in AD brain (Thomas *et al.,* 2015). Furthermore, increased expression of VEGF has been observed under hypoperfusion in the elderly brain and also in astrocytes and perivascular deposits of AD brain (Hohman *et al.,* 2015). On the contrary, decrease in the serum levels of VEGF has also been found to be associated with AD (Mateo *et al.,* 2007).

Another angiogenic factor known as basic fibroblast growth factor (bFGF) formed aggregates in association with Aβ peptides and NFTs in areas of tissue damage in AD. Further, it has been reported that NFTs of AD brain, that is supposed to be secondary to Aβ aggregation, contain heparan sulphate proteoglycans. These proteoglycans act as substrates for keen binding to bFGF (Vagnucci AH and Li 2003). Interestingly, the blood clotting factor thrombin which aggregates within vessels lining the walls of AD brain, elevated VEGF activity under hypoxia. Thrombin promotes vascular endothelial cells to release AβPP which, in turn, leads to Aβ agglomeration and reactive oxygen species (ROS) generation via activation of protein kinase C (PKC)-dependent signaling (Krenzlin *et al.,* 2016; Tripathy *et al.,* 2013). The pathological activation of the brain vasculature may also contribute to noxious mediators such as HIF-1α, angiopoietin-2 (Ang-2), matrix metalloproteinase-2 (MMP-2) and caspase 3 which lead to neuronal injury and consequent neuronal death (Grammas *et al.,* 2011). The angiogenic activity is also stimulated by numerous inflammatory mediators found in AD brain. These inflammatory mediators include tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and monocytes chemoattractant protein-1(MCP-1) which is activated by several mechanisms such as neuronal death, peroxidative and free radical injury of microvessels

and Aβ binding of the C1q component of the complement cascade (**Figure 1.3**) (Wyss-Coray and Rogers 2012).

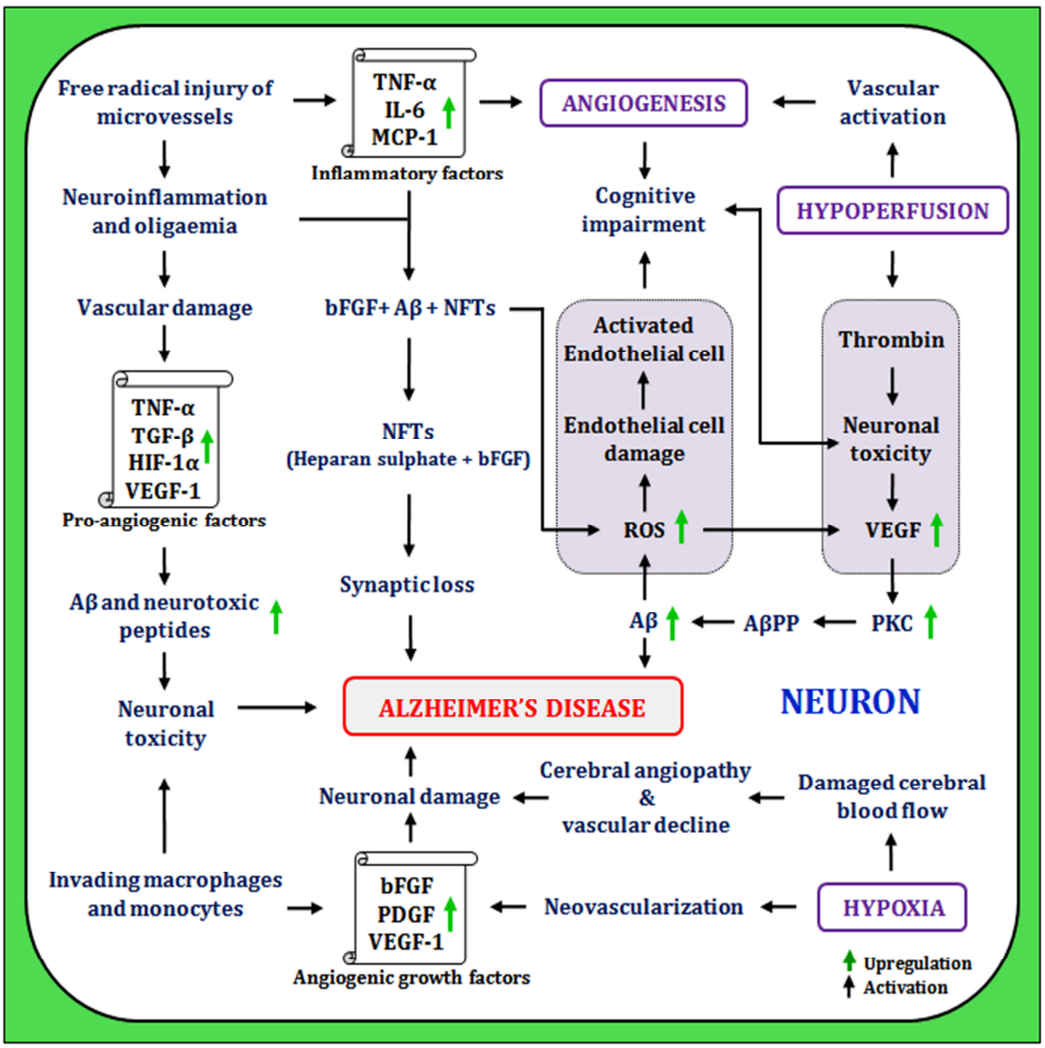


Figure 1.3: Hypoxia induced pathological angiogenesis in AD. Free radical injury of microvessels under hypoxia causes Neuroinflammation and oligaemia which thereafter leads to Aβ accumulation through vascular damage and the activation of pro-angiogenic factors (TNF-α, TGF-β, HIF-1α and VEGF-1). Further, invading macrophages and monocytes causes neuronal damage via activation of numerous angiogenic growth factors, including, bFGF, PDGF and VEGF-1. While, neuronal damage under hypoxic state is also triggered in association with both neovascularization and cerebral angiopathy. Further, hypoperfusion also contributes to cognitive impairment by activating thrombin protein. The activated thrombin directs endothelial cell damage through VEGF activation following PKC signaling cascade and leads to Aβ accumulation. TNF-α, tumor necrosis factor; IL-6, Interleukin-6; MCP-1, monocyte chemotactic protein-1; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; NFTs, neurofibrillary tangles; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; AβPP, amyloid-β Protein Precursor; PKC, protein kinase C

Additionally, invading macrophages and monocytes also secrete angiogenic growth factors such as VEGF, bFGH and platelet-derived growth factor (PDGF) which are supposedly involved in AD (Vagnucci AH and Li 2003; Dalton *et al.,* 2013). Currently, a new terminology has been assigned to angiogenesis in AD; pathogenic neoangiogenesis. It likely occurs in redeeming response to damaged cerebral blood flow and causes BBB leakage in AD. This BBB leakage was brought about by amyloidogenesis that renders a strong connect between cerebrovascular angiopathy and AD. Moreover, the "vascular hypothesis'' represents that BBB disruption and leakage in AD is perhaps due to hypoxia and neuroinflammation contributing to vascular decline and apoptosis (Biron *et al.,* 2013).

1.5.2 Tau metabolism: An interlink between hypoxia and Alzheimer's disease

The etiology of AD is signified by spatial memory impairment which, in turn is associated with elevated tau phosphorylation. While tau plays an important role in maintaining the structural integrity of microtubules, it can also direct the disruption of microtubule network once it becomes hyperphosphorylated by various kinases such as, microtubule affinity regulating kinase (MARK), glycogen synthase kinase (GSK), mitogen-activated protein kinase (MAPK) and PKC leading to the formation of NFTs (Kumar *et al.,* 2015). In AD, the hyperphosphorylation of tau weakens its ability to bind the microtubules which results in reduced stability and leads to AD pathogenesis (Tournissac *et al.,* 2017). Under hypoxic condition, tau was phosphorylated at Ser198, Ser199, Ser202, Ser262, Ser396, Ser404 and Thr205 in the hippocampus of AD brain. Moreover, the levels of Tyr216-phosphorylated glycogen synthase kinase-3beta (GSK-3β) and Tyr307-phosphorylated protein phosphatase 2A (inactivated form of PP2A) increased significantly, whereas, the levels of methylated PP2A (activated form of PP2A) were decreased in the hippocampus upon hypoxia (Zhang *et al.,* 2014). Furthermore, the cerebral hypoperfusion (a deficiency in the amount of blood in the body, a state "oligemia") is also found to be associated with a significant increase in the

tau phosphorylation at Ser 212, Thr 214, thereby having profound and long-lasting effects on tau activity (Koike *et al.,* 2010). Further, acute hypoxic exposure also evokes extracellular signal–regulated kinase (ERK) signaling cascade in tau phosphorylation that leads to the formation of NFTs, hence associated with the pathology of AD (Fang *et al.,* 2010). Recently, chronic cerebral hypoperfusion has also been reported to enhance tau hyperphosphorylation and reduces autophagy in AD mice (Qiu *et al.,* 2016). In a study serum tau protein level was measured in order to predict neurological outcome following resuscitation from hypoxic brain injury. Interestingly, early elevated tau protein came out within 24h of cardiac arrest due to hypoxia, while delayed elevations became visible after 24-48h (Randall *et al.,* 2013). This outcome depicted the tau release kinetics for hypoxic brain injury estimation and forecast of cerebral function outcome.

The hypoxia-induced raise in the tau phosphorylation is also linked with a considerable increase in the generation of p35, p25 and calpain (**Figure 1.4A**). This signifies that hypoxia induces aberrant cyclin dependent kinase-5 (CDK5)/p25 activation via calpain upregulation, accelerates Aβ pathology and possibly contributes to the hypoxia-mediated behavioral change in AD patients (Gao *et al.,* 2013). Calpain is a protein belonging to the family of calcium-dependent, proteolytic enzymes, which is expressed ubiquitously in mammals and many other organisms. The hypoxia-triggered abnormal calpain activation may also enhance ER stress-induced apoptosis in AD brain. The ER stress triggers an unfolded protein response (UPR), leading to the tau hyperphosphorylation associated neuronal death. However, In APPswe cells, m-calpain silencing reduces hypoxia-induced cellular dysfunction that results in the suppression of GSK3β activity, ER stress, tau hyperphosphorylation and caspase activation (Wang *et al.,* 2013). A few studies have reported the effect of hypobaric hypoxia on tau phosphorylation. The level of tau in the hippocampus was elevated subsequent to hypobaric hypoxia exposure and associated with memory dysfunction and cognitive impairment (Chen *et al.,* 2014). Recently, asparaginyl endopeptidase has been implicated in the tau hyperphosphorylation since its (activated form) level is significantly increased under hypoxic insult. Under

hypoxia, PP2A protein, responsible for tau dephosphorylation, is cleaved by the activity of this enzyme at Asn-175 into the N-terminal fragment (I2NTF) and the C-terminal fragment (I2CTF). Further, both I2NTF and I2CTF are involved in binding to the catalytic subunit of PP2A and inhibit its activity, thereby leading to the hyperphosphorylation of tau in AD (Basurto-Islas *et al.,* 2013).

1.5.3 Amyloid beta (Aβ): A major culprit of hypoxia mediated Alzheimer's disease

Aβ peptides play a crucial role in AD progression. Normally, these peptides are generated by many cell types in the body; however, their expression is particularly upraised in the brain. The accruement of Aβ forms the pathological hallmark of AD in the brain, i.e. the extracellular plaques (Watts and Prusiner 2017). The toxic Aβ peptide formed by brain amyloidosis is driven by mutations in the genes encoding for AβPP or γ-secretase (Crews *et al.,* 2010). Further, both *in vitro* and *in vivo* studies have clearly observed that hypoxic stress elevates the β-secretase cleavage activity and beta-secretase 1 (BACE1) gene transcription of AβPP for Aβ production (**Figure 1.4B**) (Cole and Vassar 2008). Interestingly, hypoxia treatment significantly and markedly increased Aβ accumulation, neuritic plaque formation and potentiated memory loss in a Swedish mutant AβPP transgenic AD mice model (Sun *et al.,* 2006). Similar to hypoxia, mild transient brain hypoperfusion has shown an acute increase in Aβ production through induced β-secretase, which in turn is regulated by HIF-1α (Koike et al., 2010). Indeed, hypoxia is a direct outcome of hypoperfusion, a most ordinary vascular component among AD risk factors (Sun *et al.,* 2006). Moreover, hypoxic insult is also responsible for triggering oxidative stress, which is required for proper activation of γ-secretase and BACE1 (Salminen *et al.,* 2017). Hypoxia associated with hypoperfusion causes the mitochondrial ETC to generate higher levels of ROS which in turn leads to the oxidative stress. The oxidative stress thus leads to BACE1 and γ-secretase activation, AβPP cleavage and subsequent generation of $A\beta_{42}$ (Tamagno *et al.,* 2012). Studies have revealed that expression of zebrafish AD-related genes such as;

BACE1, PS1, presenilin-2 (PS2) appa and appb are increased under hypoxia exposure. These genes have high propensity to be involved in the catalytic process of AβPP (Newman *et al.,* 2014). Under hypoxia, a dominant negative form of PS1 putatively blocks γ-secretase activity in order to prevent BACE1 upregulation thereby showing negative impact of PS1 on γ-secretase activity (Moussavi Nik *et al.,* 2013). Moreover, PS1/2 activity is required for proper HIF activation. Interestingly, PS1/2 mutations involving the generation of amyloid precursor protein intracellular domain (AICD) have been identified in AD patients differentially affected with the hypoxic response. Further, presenilin (PS) is also involved in the regulation of the oxygen sensing pathway via modulation of PHD proteins (Kaufmann *et al.,* 2013). PHDs are key regulators of hypoxic signaling responsible for proper regulation of HIF-1α. Furthermore, hypoxia induced ischemic stroke has been reported to be associated with AD (Lucke-Wold *et al.,* 2015). Interestingly, in aging brain it has been found that inflammation along with ischemia/hypoxia produces plaque-like aggregates of myelin, Aβ and AβPP which aggravates the brain injury (Zhan *et al.,* 2015).

It has also been reported that apolipoprotein E (ApoE), a risk factor of AD is associated with cerebral ischemia. Neuronal ApoE was significantly increased in all ischemic groups when compared with controls in an AD mice model. In response to ischemia, the level of ApoE along with $A\beta_{1-40}$, $A\beta_{1-42}$ was increased in the CA1 and CA3 neurons (Qi *et al.,* 2007). Furthermore, studies have outlined a link between hypertension and AD in individuals with ApoE genotype through brain hypoperfusion and hypoxia. This might accelerate Aβ aggregation in the brain that disrupts cell-to-cell connectivity and leads to neuronal cell death (Feldstein 2012). In a study, when pregnant APP (Swe)/PS1 (A246E) transgenic mice was exposed to high-altitude hypoxic exposure, the prenatal hypoxic mice demonstrated a remarkable deficit in spatial learning and memory with a significant decrease in synapses (Zhang *et al.,* 2013). Moreover, significantly higher level of AβPP, lower level of the Aβ-degrading enzyme neprilysin (NEP) and increased Aβ agglomeration was observed in the brain of prenatal hypoxic mice (Kerridge *et al.,* 2015).

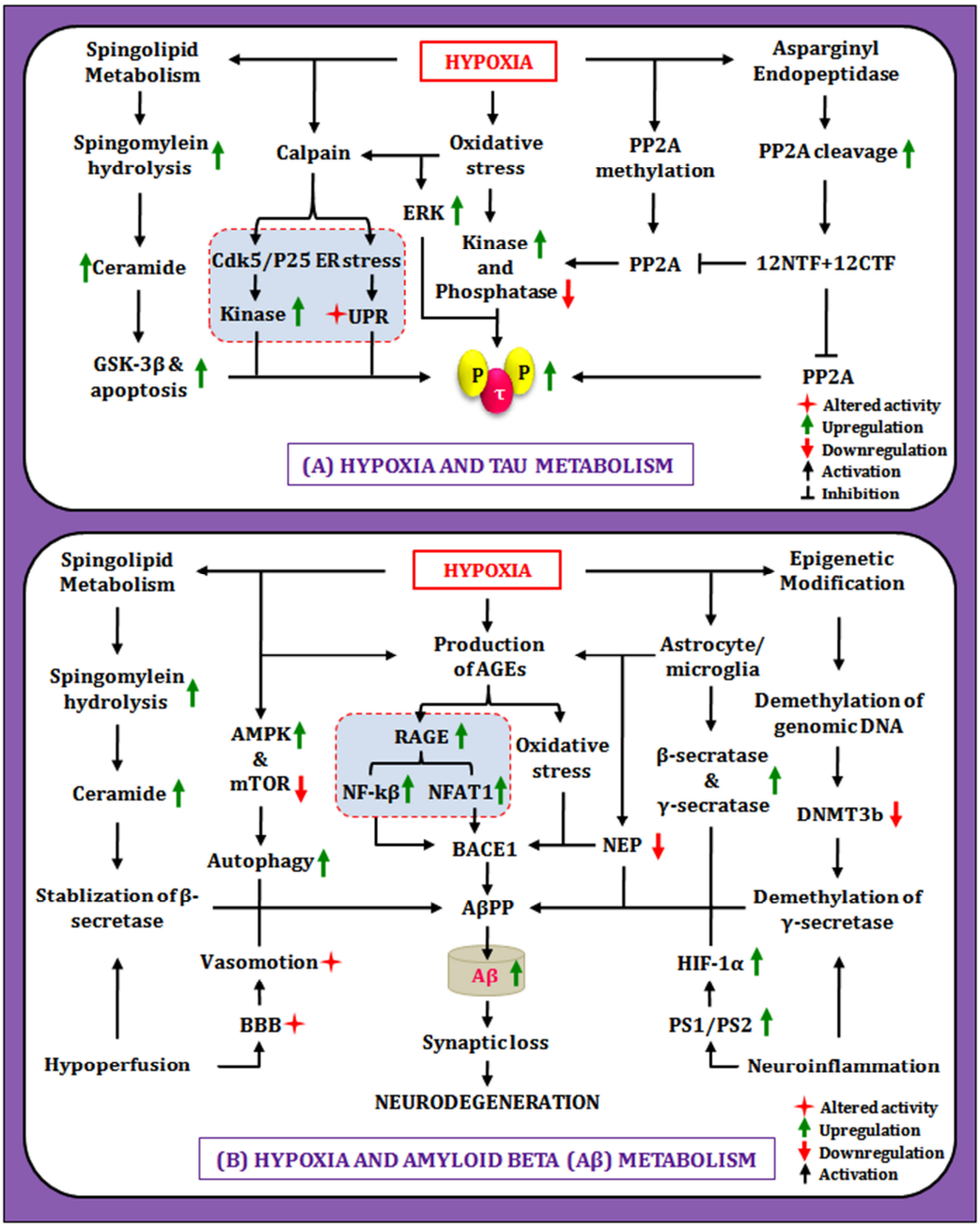


Figure 1.4: Signaling cascades associated with hypoxia induced tau and amyloid beta (Aβ) metabolism. (A) Hypoxia and tau metabolism: The process of tau phosphorylation under hypoxia is triggered by various factors. Firstly, spingomylein hydrolysis leads to an increased ceramide level in the brain that further contributes to the activation of GSK-3β & apoptosis, thereby causing tau phosphorylation. Further, calpain activation under hypoxic stress causes tau phosphorylation via activation of Cdk5/P25 and ER stress. Since, activated Cdk5/P25 promotes kinase activity and ER stress leads to an altered UPR response. Similarly, methylation in PP2A gene and upregulation of ERK protein induced by hypoxia also lead to tau phosphorylation via activation of kinase. At last, activation of Asparginyl Endopeptidase under hypoxia also acts as a central player in tau phosphorylation. Since, peptidase activity of this enzyme cleaves PP2A

into two fragments, which further block the phosphatase activity of PP2A and lead to abnormal tau phosphorylation. **(B) Hypoxia and amyloid beta (Aβ) metabolism:** The process of Aβ metabolism under hypoxia is triggered by various factors. Firstly, spingomylein hydrolysis leads to an increased ceramide level in brain that further contributes to the stabilization of β-secretase, thereby causing Aβ accumulation. Similarly, the activation of AMPK-mTOR signaling under hypoxia directs Aβ accumulation by promoting autophagy. Additionally, production of AGEs during hypoxia contributes towards toxic Aβ formation via BACE1 Upregulation. Moreover, AGEs also act as an activator of RAGE which further activates its downstream molecules, including NF-κβ and NFAT1 and leads to the upregulation of BACE1 crucial for Aβ formation. Likewise, Microglial activation under hypoxic stress also leads to Aβ formation by activating both β-secretase & γ-secretase. Epigenetic modification of DNMT3b during hypoxia contributes to Aβ accumulation. Since, downregulated expression of DNMT3b under hypoxia causes demethylation of γ-secretase thus leads to the formation of toxic Aβ. Similarly, hypoperfusion activates Aβ formation via impaired BBB and vasomotion. Finally, Neuroinflammation is also accountable for triggering Aβ formation via HIF-1α activation associated with PS1/PS2 upregulation. GSK-3β, glycogen synthase kinase-3β; Cdk5, cyclin-dependent kinase 5; UPR, unfolded protein response; ERK, extracellular signal–regulated kinase; PP2A, protein phosphatase 2; NTF, N-terminal fragment; CTF, C-terminal fragment; AMPK, AMP-activated Protein Kinase; BBB, blood–brain barrier; AβPP, amyloid-β Protein Precursor; BACE1, beta-secretase 1; NEP, neprilysin; RAGE, receptor for advanced glycation end products; NF-κβ, f; NFAT1, nuclear factor of activated T-cells 1; AGEs, advanced glycation end products; DNMT3b, DNA methyltransferase 3 beta; PS1, Presenilin 1; PS2, Presenilin 2; HIF-1α, hypoxia-inducible factor 1-α

Strikingly, increased tau phosphorylation, decreased HIF and enhanced activation of astrocytes and microglia were also observed under hypoxic exposure (Zhang *et al.,* 2013). These observations have signified the possible impact of hypoxia on memory exiguity and consequent neuronal death. Cerebral amyloidangiopathy (CAA), a cerebrovascular disorder frequent in AD, has been characterized by the agglomeration of Aβ peptide in cerebral blood vessel walls. CAA is associated with a loss of vascular integrity that results in impaired regulation of cerebral circulation and augmented cerebral ischemic susceptibility. Further, vasomotion which is responsible for spontaneous rhythmic modulation of arterial diameter is typically observed in arteries/arterioles in various vascular beds, including the brain and is liable to participate in tissue perfusion and oxygen delivery regulation. Under hypoperfusion and hypoxia, vasomotion is impaired and provokes the accumulation of Aβ in cerebral blood vessel walls with loss of vascular integrity that results in altered cerebral circulation (Di Marco *et al.,* 2015). Various *in vivo* and *in vitro* studies have depicted the involvement of CIH and IH in obstructive sleep apnea (OSA) associated with AD patients. CIH and IH significantly increased the levels of $A\beta_{42}$ but not $A\beta_{40}$ in the brains of mice without the increase in HIF-1α expression.

These observations suggest the potential role of CIH induced OSA in intensifying the advancement of AD (Shiota *et al.,* 2013).

1.5.4 Unconventional players in hypoxia mediated Alzheimer's disease

Although hypoxia has direct involvement in tau and Aβ metabolism, several other factors have also been found to affect tau and Aβ metabolism under hypoxia. Chronic hypoxia has been shown to mediate several epigenetic modifications in AD. It aggravated AD pathogenesis through demethylation of genes encoding for β- and γ-secretase by downregulation of DNA Methyltransferase 3 Beta (DNMT3b) thereby leading to Aβ accumulation in neurons (Liu *et al.,* 2016). Further, the activity of cholinesterase enzymes acetylcholinesterase (AChE) and butyryl cholinesterase (BChE) which promotes growth in the brain also gets affected due to hypoxia at different stages of ontogenesis. This leads to changes in brain development and formation of behavioural reactions, learning and memory and also increases the risk of sporadic form of AD (Kochkina *et al.,* 2015). Moreover, cerebral hypoxia provokes a large number of inflammatory proteins in the brain endothelial cells via various signaling cascades. The p38 MAPK signaling cascade has been implicated in endothelial injury and inflammation. Exposure of brain endothelial cells to hypoxia showed a time-dependent increase in pp38 MAPK. The expression levels of Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 **(**COX-2) are significantly elevated under hypoxic exposure contributing to AD (Grammas *et al.,* 2011). Another factor contributing to AD under hypoxia is the formation of bundles of filaments containing 1∶1 cofilin∶actin, which weaken transport and synaptic function. Cofilin-actin rods provide a mechanism for synapse loss bridging the amyloid and pro-inflammatory cytokines (TNF-α, interleukin-1beta (IL-1β) and IL-6) hypotheses for AD under hypoxic insult (Walsh *et al.,* 2014). Studies also reveal that continuous O_2 supply is needed for normal operation of the brain, even in the resting-state. Hypoxia increases resting-state electroencephalographic (EEG) rhythms in the delta range and decreases those in the alpha range with a pattern similar to that observed in AD (Vecchio *et al.,* 2012). Accumulating evidence indicates that under brain hypoxia and reduced glucose availability, the concentration of ATP and 2, 3-

diphosphoglycerate (2,3-DPG) is reduced which in turn, generates endogenous ammonia; a potential neurotoxin of AD brain (Kosenko *et al.,* 2014).

Another important candidate, granular non-fibrillar aggregates (GNAs) are also identified as a possible toxic species in AD. As a consequence of acidic environment (hypoxic condition), GNAs are formed on the surface of negatively charged biological membranes. Further, the $A\beta_{1-40}$ -GNAs complex disturbs the bilayered structure of model membranes and seems to be more toxic to cells with negatively charged membranes (Benseny-Cases *et al.,* 2012). The genome-wide association studies (GWAS) indicated the role of hypoxia in mitochondrial F0 (ATP5H)/Potassium channel tetramerization domain-containing protein 2 (KCTD2) loci. This channel is involved in mitochondrial energy production and neuronal hyperpolarisation and has novel association with AD (Boada *et al.,* 2014). Recently, increase in ceramide content has been observed in AD brain, which directly stimulates biochemical changes in the brain associated with both tau hyper-phosphorylation and Aβ accumulation. Under ischemia/hypoxia, the ceramide content is increased, as a consequence of sphingomyelin hydrolysis. Further, high ceramide concentration induces mitochondria-dependent neuronal apoptosis, aggravate ROS production, curtail ATP level, release cytochrome c and activate caspase-3 (Car *et al.,* 2012). The normal functioning of proline isomerase, Pin1 is essential for protecting against age-dependent neurodegeneration in AD. Studies have reported the involvement of hypoxic insult in the oxidization state of proline isomerase Pin1. Hypoxia led to Pin1 inactivation through Cys113 oxidation and thus promoted turnover of tau and AβPP proteins *in vitro* (Chen *et al.,* 2015). Likewise, traumatic brain injury (TBI) has currently been associated as an epigenetic risk factor for AD. TBI has been found involved in the axonal injury and hypoxia associated with cognitive impairment in AD. Additionally, epidemiological studies also showed that 30% of patients, who died of TBI, had Aβ plaques responsible for AD (Sivanandam and Thakur 2012). The involvement of hypoxic insult in AD has also been proven by *in vitro* O_2 glucose deprivation treatment study in rat brain capillary endothelial cells (RBE4). Where, hypoxia elicited $A\beta_{42}$ peptide production upto 250% through HIF-1α mediated BACE1 up-regulation (Bulbarelli *et al.,* 2012).

1.6 HYPOXIA AND OTHER NEURODEGENERATIVE DISORDERS

1.6.1 Parkinson's disease (PD)

Parkinson's disease (PD) is a progressive neurodegenerative disorder in which altered mitochondrial dynamics has been shown to play a crucial role in its pathogenesis (Perier and Vila 2012). Impairment in mitochondrial dynamics causes reduced O_2 consumption and subsequent activity of prolyl hydroxylase, which in turn degrades HIF-1α (Speer *et al.,* 2013). Hao *et al* has suggested that any changes in the BBB permeability under hypoxia could be one mechanism permitting endotoxin into the brain and lead to idiopathic PD (Lange *et al.,* 2016; Hao *et al.,* 2016). Further, involvement of both silent information regulator 1 (SIRT1) and HIF-1α have also been reported to play a cardinal role in the PD pathophysiology. In methyl-4-phenylpyridinium (MPP+) treated PD models, the expression of HIF-1α and its target factors vascular endothelial growth factor A (VEGFA) and lactate dehydrogenase A (LDHA) were found to be increased and that SIRT1 expression was found to be inhibited. Moreover, acetylation of histone H3 lysine 14 (H3K14) along with the HIF-1α was increased when SIRT1 was knocked down; suggesting that SIRT1 was involved in the epigenetic regulation of HIF-1α, thus providing a link between PD and SIRT1/HIF-1α signaling (Dong *et al.,* 2016). Further, patients with CIH and IH induced OSA were found to have an increased risk for developing PD (Yeh *et al.,* 2016). Deficiency in DJ-1 (PARK7) is another cardinal player in the pathology of PD. Interestingly, mutations in DJ-1 promoted VHL mediated degradation of the α-subunit of HIF-1α which further leads to MPP+-induced toxicity in neurons (**Figure 1.5**) (Parsanejad *et al.,* 2014). Likewise, loss of PTEN-induced putative kinase 1 (PINK1) has also been reported to attenuate HIF-1α induction by preventing 4E-BP1-dependent switch in protein translation under hypoxia and thereby leading to PINK1-associated PD pathogenesis (Lin *et al.,* 2014). The association between ATP13A2 (PARK9) and HIF-1α has pointed out the involvement of hypoxia in PD. Further, the promoter region of human PARK9 gene, the gene linked to a rare juvenile form of PD contains hypoxia response elements, which can bind to transcription factor HIF-1α. The upregulated PARK9 transcription via HIF-1α under

hypoxia has been observed in HEK293 and dopaminergic MN9D cells. Moreover, knockdown of PARK9 has been found to abrogate cellular iron homeostasis and neuronal viability elicited by prolyl hydroxylase domain-containing protein 2 (PHD2) inhibitions *in vivo* and in cultured dopaminergic cells under conditions of mitochondrial stress. Mechanistically, this is due to the role of PARK9 in maintaining lysosomal iron stores. This further constitutes a novel mechanism by which alterations in PARK9 activity may contribute to drive PD-related neuropathology (Van Veen *et al.,* 2014). Recently, HIF-1α ortholog Similar (Sima) which is associated with mitochondrial retrograde signaling has been reported to cause neuronal dysfunction in Drosophila model of familial PD (Duncan and Bateman 2016; Cagin *et al.,* 2015). Further research is required to identify plausible mechanisms involved between hypoxia and PD.

1.6.2 Amyotrophic lateral sclerosis (ALS)

ALS is a devastating brain disorder involving progressive degeneration of motor neurons in motor cortex, spinal cord and brainstem (Pronto-Laborinho *et al.,* 2014). The clinical course of the degenerative motor neuron disorder is closely associated with hypoxia. The normal response to hypoxia involves two crucial cascades, in particular: the HIF-1α cascade and the nuclear factor NF-κB pathway (responsible for the production of inflammatory mediators). Any alterations in these pathways contribute to the fatal complication of ALS. Defects in VEGF gene expression and deregulation of the HIF-1α pathway are significantly impaired in ALS patients. Moreover, impaired HIF-1α activation linked with low levels of proteolysis by PHD2 has been found in sporadic ALS patients (Moreau *et al.,* 2011). Further, respiratory insufficiency and hypoxemia are closely linked with the clinical course of ALS. Chronic respiratory insufficiency and hypoxemia generally occur late in the disease course, but rapid episodes of intermittent hypoxemia followed by reoxygenation can occur early and insidiously. Selective defect of HIF-1α mediated angiogenic factors such as angiogenin (ANG) has been reported in sporadic ALS patients, compared to age matched controls. Further, early activation of the NF-κB pathway, IL-6, COX-2, PGE2, TNF-α and angiopoietin-2 (Ang-2) were also found in the same cohort of sporadic ALS patients (**Figure 1.5**) (Moreau *et al.,* 2010).

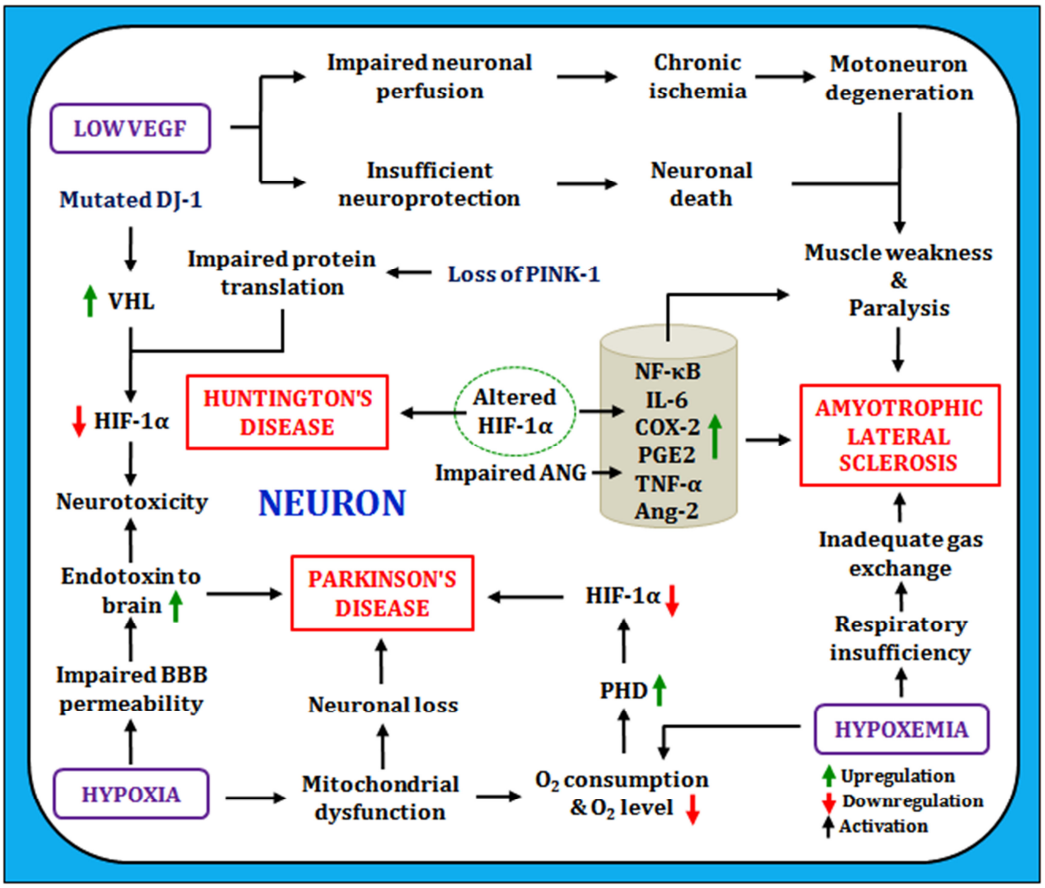


Figure 1.5: Signaling cascades associated with hypoxia mediated NDDs. Low VEGF levels alter spinal cord perfusion and cause chronic ischemia of motoneurons, but also deprive these cells of VEGF-dependent survival and neuroprotective signals. Both phenomenona results in the progressive degeneration of motoneurons as a typical symptom of ALS. Similarly, selective defect of HIF-1α mediated angiogenic factors such as ANG causes sporadic ALS. Further, early activations of the NF-κB, IL-6, COX-2, PGE2, TNF-α, and Ang-2 are also found in the same cohort of sporadic ALS patients. Hypoxemia, another cause of ALS contributes to respiratory insufficiency in ALS patients. While in case of HD, altered or low HIF-1α levels have been observed. Further, hypoxia also causes impaired BBB permeability, which allows endotoxin into the brain and lead to idiopathic PD. Likewise, mitochondrial dysfunction under hypoxia causes reduced O_2 consumption, which further promotes PHD activity to degrade HIF-1α and lead to PD. In similar fashion, mutations in DJ-1 promoted VHL mediated degradation of the α-subunit of HIF-1α which further leads to MPP+-induced toxicity in neurons. Further, loss of PINK1 attenuates HIF-1α induction by preventing 4E-BP1-dependent switch in protein translation under hypoxia and thereby leading to PINK1-associated PD pathogenesis. NDDs, neurodegenerative disorders; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease; PD, Parkinson's disease; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau; HIF-1α, hypoxia-inducible factor 1-alpha; BBB, blood-brain barrier; PHD, Prolyl-4-hydroxylase-domain; ANG, angiogenin; PINK-1, PTEN-induced putative kinase 1; NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor; IL-6, Interleukin-6; Ang-2, angiopoietin-2

Importantly, impaired cytoplasmic-nuclear transport of HIF-1α in the cytoplasm of anterior horn cells (AHCs) has been shown in mutant superoxide dismutase 1 (mSOD1) transgenic mice. Further, transport of cytoplasmic HIF-1α to the nuclear envelope and into the nucleus is altered from the presymptomatic stage in ALS patients. Thus, impaired cytoplasmic-nuclear transport of HIF-1α might precede motor neuron degeneration in ALS (Nagara *et al.,* 2013). Moreover, respiratory muscle weakness in ALS causes ventilatory insufficiency and tissue hypoxia, which triggers several metabolic pathways and increases EPO synthesis in particular. EPO is a glycoprotein which imparts neuroprotection through erythropoiesis. Interestingly, EPO levels were markedly upregulated in ALS patients with respiratory impairment (Carilho *et al.,* 2011). Recently, involvement of nocturnal hypoxia in ALS has been found to be associated with cognitive dysfunction. However, the exact mechanism behind such alterations is still unknown (Park *et al.,* 2013).

1.6.3 Huntington's disease (HD)

Huntington's disease (HD*)* is a fatal progressive, genetic neurodegenerative disorder manifested by emotional imbalance, cognitive decline and loss of movement co-ordination caused due to an autosomal dominant mutation in huntingtin gene (HTT) (Nopoulos 2016). The pathological role of hypoxia in HD is still unrevealed but reduced HIF-1 expression has been reported in case of HD (**Figure 1.5**). The research on HTT induced mitochondrial dysfunction provides an evidence for the role of hypoxia in HD. Since, hypoxia induced HIF-1 regulates glycolysis and HMP shunt pathway, which augments the generation of ATP and NADPH for cell survival. Therefore, HTT induced altered mitochondrial dynamics makes an alternate strategy for compensating mitochondrial energy deficits thus lead to neuronal survival (Zhang *et al.,* 2011). Currently, chemical preconditioning with 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase (SDH) has been observed to augment tolerance against experimental hypoxia in the brain, thus provide another evidence for a

link between hypoxia and HD. The reduced levels of the mitochondrial enzyme SDH are observed in the post mortem brains of HD patients and energy metabolism defects have also been reported in both presymptomatic and symptomatic HD patients (Turan *et al.,* 2008). Further, a deeper knowledge on the role hypoxia in HD pathology will bring new insights into the molecular pathways underlying the mechanisms associated between hypoxia and HD.

1.7 THERAPEUTIC STRATEGIES

Neuronal dysfunctions associated with hypoxia mediated injuries have currently emerged as a challenging task for neurobiologists. Impaired response of the brain to hypoxia and disrupted HIF-1 signaling represent pathological events that occur during the course of NDDs. Such pathological events lead to altered BBB permeability, reduced ATP production and proinflammatory cytokines release thereby leading to neuronal damage (Peers *et al.,* 2009). Although an effective therapeutics is still lacking, but numerous therapeutic strategies are currently devised to attenuate the toxicity associated with hypoxic stress. For instance, numerous biomolecules/drugs based therapy has been designed to curtail hypoxia induced neuronal dysfunction. Similarly, various other therapeutic approaches have been recently identified to mitigate the pathophysiology associated with hypoxic insults in neuronal damage.

1.7.1 Therapeutic interventions of biological compounds against hypoxia induced neuronal dysfunction

Owing to the multifactorial nature of hypoxia induced neuronal dysfunction, it is important to combine different compounds/molecules to recoup normal functioning of the brain. For this reason, a broad range of biological compounds are being investigated to alleviate hypoxic stress associated toxicity in NDDs. For instance,

supplementations of vitamin B6/B12/folate+choline significantly improve hypoxia-induced memory deficits by dint of attenuating tau hyperphosphorylation at multiple AD-related sites. This effect was brought about by upregulating the activity of inhibitory Ser9-phosphorylated GSK-3β (Yu *et al.,* 2016). Similarly, the neuroprotective effect of Humanin (HN) has been reported to attenuate the toxicity associated with hypoxia in retinal ganglion cell 5 (RGC-5) cells (Men *et al.,* 2012). In like manner, administration of lutein also protected against either $CoCl_2$-induced chemical hypoxia or H_2O_2-induced oxidative stress in RGC-5 cells (Li and Lo 2010). Also, exposure of the brain to mild hypoxic episodes has been shown to increase brain tolerance to severe hypoxia, a mechanism called preconditioning. Such preconditioning also reduced the levels of AβPP and its degradation in the brain (Sheldon *et al.,* 2014). Since elevated thrombin level in the brain and cerebral microvasculature, is neurotoxic in AD, thrombin inhibitors prevent against these effects in AD transgenic mice. In this line, thrombin inhibitors such as dabigatran can be considered as potential therapeutics against dementia (Grammas and Martinez 2014). Similar to AD, numerous compounds are used for alleviating hypoxia associated toxicity in PD. For instance, Orexin-A is a neuropeptide secreted by hypothalamic neurons, which shows neuroprotection in many neurological conditions, including cerebral ischaemia. Treatment with Orexin-A not only induces HIF-1α but also activates its downstream targets such as VEGF and EPO, which are further accountable for attenuating MPP+-induced cell injury (Feng *et al.,* 2014). Moreover, intranasal administration of Deferoxamine (DFO) has also depicted potential drug activity in treating NDDs and other psychiatric disorders in which GSK-3β and HIF-1α play a prominent role (Fine *et al.,* 2012). DFO-mediated up-regulation of HIF-1α and VEGF prevented dopaminergic neuronal death via activation of the ERK and p38MAPK signaling in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mice. DFO treatment efficiently attenuated behavioral deficits and increased neuronal survival, in the substantia nigra **(**SN) and striatum (Guo *et al.,* 2016). It has been found that inhibition

of iron-dependent enzyme class PHDs by 3, 4-dihydroxy benzoate (DHB) shows protection against MPTP-induced nigral dopaminergic cell loss and promotes HIF-1α expression within these neurons. PHDs target α-subunits of HIF proteins for proteasomal degradation that contribute to stroke and hypoxic-ischemic injuries (Siddiq *et al.,* 2007). Another compound which reportedly showed protection against hypoxic injuries is M30. M30 5-(N-methyl-N-propargylaminomethyl)-8-hydroxy quinoline] is an iron chelator which is also a selective monoamine oxidase (MAO) AB inhibitor in the brain. In PD, M30 induced HIF expression which in turn triggered the production of brain-derived neurotrophic factor (BDNF), VEGF, EPO and glia-derived neurotrophic factor (GDNF) and thus imparted neuroprotection. Further, M30 displayed *in vitro* and *in vivo* neuroprotective activity in other NDDs. Similarly, Ladostigil is a compound which protected against ischemia induced cytotoxicity in cultured neuronal cells. Additionally, the neurorestorative activity of both these drugs is related to HIF activation upon PHDs inhibition (Youdim 2013). Recently, Uridine prodrug PN401 has been identified as a potential drug in diminishing toxic effects of chemical hypoxia and ceramide. Besides, it also reduced hydrogen peroxide induced ROS formation and mitochondrial DNA damage in neurons (Saydoff *et al.,* 2013). Furthermore, preconditioning of rat C6 astroglial cells with HIF-1 inducer $CoCl_2$ exerted neuroprotection against 3-NP induced metabolic stress in HD (Yang *et al.,* 2005). Likewise, administration of *Naringenin* and *Quercetin* also reversed the effect of hypobaric hypoxia and elicited neuroprotective response in the murine model. The expression of HIF-1α, VEGF, active caspase 3 and ubiquitin levels were significantly reduced upon drug treatment. However, the moderate expressions of chaperones (Hsp90, Hsp70 and C-terminus Hsp70 interacting protein (CHIP)) were reported (Sarkar *et al.,* 2012). Likewise, many other compounds which have been reported in attenuating hypoxic injuries in the brain have been illustrated in **Table 1.2**.

Table 1.2: Different drugs and biomolecules to attenuate the hypoxic insult in brain

S.No.	Compounds	Involved factors/ proteins	Associated mechanisms	Principle phenotypes	References
1	Curcumin	Prdx6 and NF-κB	It curtails hypoxia-induced cell death by preventing NF-κβ induced repression of peroxiredoxin-6 in mouse hippocampal cells.	AD	(Chhunchha *et al.,* 2013)
2	Vitamin B6/B12	Tau and GSK-3β	It improves hypoxia-induced memory deficits, by dint of attenuating tau hyperphosphorylation at multiple AD-related sites through upregulating inhibitory Ser9-phosphorylated GSK-3β.	AD	(Yu *et al.,* 2016)
3	Valproic acid (VA)	NEP	Administration of VA restored NEP activity and memory deficit caused by prenatal hypoxia in adult human neuroblastoma SH-SY5Y cells.	AD	(Nalivaeva *et al.,* 2012)
4	Telmisartan	iNOS and NO	It inhibits excessive iNOS, NO generation, reduced lipid peroxidation and inflammatory responses. Besides, it also improves the hippocampal apoptosis induced by CIH, the most characteristic pathophysiological change of OSAS.	NDDs	(Yuan *et al.,* 2015)
5	Deferoxamine (DFO)	HIF-1α, GSK-3β, VEGF, ERK and p38MAPK	DFO-mediated up-regulation of HIF-1α and their associated growth factors, including VEGF have reported to inhibit dopaminergic neuronal death via the activation of the ERK and p38MAPK signaling in MPTP-treated mice. Further, it also protects against rotenone-induced neuronal loss by two interconnected mechanisms, the accumulation of HIF-1 and the induction of autophagy via HIF-1.	PD, AD and HD	(Guo *et al.,* 2016; Chen *et al.,* 2013)
6	Dabigatran	Thrombin	It blocks the effects of hypoxia on brain endothelial cells and curtails vascular inflammation in transgenic AD mice.	AD	(Grammas and Martinez 2014)

S.No.	Compounds	Involved factors/ proteins	Associated mechanisms	Principle phenotypes	References
7	SB203580	p38MAPK, Ang-2 and MMP-2	SB203580 curtails hypoxia-mediated increase in p38MAPK expression. Since, inhibition of p38MAPK in cultured brain endothelial cells reduces the hypoxia-induced increase in the inflammatory proteins, including Ang-2 and MMP-2 in AD.	AD	(Sanchez *et al.,* 2012)
8	Orexin-A	HIF-1α, VEGF and EPO	Orexin-A not only provokes HIF-1α but also activates downstream targets of HIF-1α including, VEGF and EPO which are further accountable for attenuating MPP+-induced cell injury.	PD	(Feng *et al.,* 2014)
9	FG0041	HIF-1	It is 2-oxoglutarate analogue which enhances HIF-1 activity and extracellular dopamine release in the striatum of the rat brain.	PD	(Johansen *et al.,* 2010)
10	3,4-dihydroxybenzoate (DHB)	HIF-1α and PHDs	It shows protection against MPTP-induced nigral dopaminergic cell loss and promotes HIF-1α expression within these neurons through inhibition of PHDs activity.	PD	(Siddiq *et al.,* 2007)
11	Clioquinol (CQ)	HIF-1	CQ treatment significantly induces HIF-1 expression to slow cognitive decline and decrease plasma A levels in AD. Additionally, *in vitro* CQ treatment also increases HIF-1 accumulation and protects against MPTP-induced nigral cell loss.	AD and PD	(Lee *et al.,* 2009; Ritchie *et al.,* 2003)
12	VK-28	HIF-1	It stabilizes altered HIF-1 stabilization and HIF-1-dependent transcriptional activation thus shows neuroprotection.	PD	(Shachar *et al.,* 2004)
13	M30	HIF, BDNF, VEGF, EPO and GDNF	M30 induced HIF expression triggered production of BDNF, VEGF, EPO and GDNF and thus imparted neuroprotection.	AD, PD and ALS	(Youdim 2013; Benkler *et al.,* 2010)

S.No.	Compounds	Involved factors/ proteins	Associated mechanisms	Principle phenotypes	References
14	HLA20	HIF-1	It augments HIF-1 expression and its nuclear translocation in motor-neuron-like NSC-34 cells, which confer protection against oxidative damage. Besides, HLA20 was also shown to extend survival and delay the onset of neurological dysfunction of SOD1G93A mutant mice of ALS.	ALS	(Kupershmidt *et al.,* 2009)
15	Vitamin D	Glutamate	Treatment with vitamin D greatly attenuated hypoxic brain damage *in vivo* and reduced glutamate excitotoxicity in the G93A mouse model of ALS.	ALS	(Gianforcaro and Hamadeh 2014)
16	Mimosine	HIF-1	Acts as a HIF-1 inducers which exerts neuroprotective effects against metabolic insults induced by 3-NP in HD.	HD	(Yang *et al.,* 2005)
17	Cobalt	VEGF and EPO	Displaces the free ferrous at the active site of hydroxylases thereby inhibiting HIF-1 hydroxylation promoting and lead to DA Synthesis and secretion. Additionally, it also causes increased production of ATP and NADPH.	PD and HD	(Yang *et al.,* 2005; Witten *et al.,* 2009; Hewitson *et al.,* 2004)

1.7.2 New perspectives for the treatment of hypoxia induced neuronal dysfunction

The use of biomolecules/drugs against hypoxic injuries has been extensively addressed. However, other therapeutics approaches have been identified recently to target hypoxia induced neuronal dysfunction. For instance, targeting Ca^{2+} channels in the cell membrane by antioxidants may prove beneficial in treating hypoxia associated with AD. Following hypoxia, there is an abrupt upsurge in calcium channels in the neurons which further causes Aβ accumulation and lead to synaptic loss. Likewise, m-calpain may be another potential target in AD therapeutics. Various findings have suggested that hypoxia induced m-calpain activation is involved in ER stress mediated AD pathogenesis (Wang *et al.,* 2013). Neuregulin1 (NRG1) is neuron-specific vertebrate globin, which protects neurons against apoptosis induced by O_2 glucose deprivation and hypoxia ischemia. Further, involvement of extracellular domain of NRG1beta1 (NRG1β1-ECD) has depicted to serve as a neuroprotection in AD likely via ErbB4-dependent activation of PI3-kinase/Akt cascade (Cui *et al.,* 2013). Currently, protective role of recombinant adeno-associated virus (rAAV) vector expressing human HIF-1α gene (rAAV-HIF-1α) has been reported to attenuate Aβ associated neurotoxicity and decreased apoptosis induced by hypoxia in cortical neurons (Chai *et al.,* 2014). Nuclear factor erythroid 2-related factor 2 (NRF2); a major component regulating antioxidant response is attenuated in AD brain. It directly regulates the alternative first exons of cluster of differentiation 36 (CD36), an important participant in oxidative and inflammatory processes. Further, intranasal administration with a lentiviral vector encoding human NRF2 has been reported to increase CD36 expression, ameliorate the weak antioxidant response induced by hypoxia and reduced Aβ accumulation (Wang *et al.,* 2014). Further, intermittent hypoxic training (IHT) has been another potential approach to ameliorate cerebral vascular function and curtail vascular risk factors (VRFs) such as cardiac arrhythmias, systemic hypertension and mental stress associated with AD. Furthermore, IHT prevents endothelial dysfunction of both cerebral and extra-cerebral blood vessels, rarefaction of the brain vascular network and loss of neurons in

the brain cortex, thereby improving memory (Manukhina *et al.,* 2016). It has been observed that oxygen treatment (OT) can also ameliorate cognitive function by altering protein expression of redox pathways in AD mice (Wang *et al.,* 2016). The asparaginyl endopeptidase enzyme inhibits dephosphorylation activity of PP2A and promotes tau phosphorylation in response to brain ischemia and hypoxia. Recent studies have shown that siRNA knockdown of this enzyme abolished its activity in SH-SY5Y cells (Basurto-Islas *et al.,* 2013).

Another potential approach to mitigate hypoxia associated toxicity is by preventing Pin1 oxidation. Under normal conditions, Pin1protected against age-dependent neurodegeneration in AD. However, Pin1 is oxidatively modified in response to hypoxia and thus losses its isomerisation and protein turnover activity for tau and AβPP (Chen *et al.,* 2015). Interestingly, immunization with Aβ peptides neutralized the amyloid trigger, leading to neo-angiogenesis and reversal of hypervascularity in Tg2576 AD mice. This process settles plaque burden, signifying that neoangiogenesis is a key feature underlying plaque generation (Biron *et al.,* 2013). Further, Normobarichyperoxia (NBO) as a tool for enhancing oxygen delivery showed neuroprotective effect by inhibiting γ-secretase cleavage of AβPP (Gao *et al.,* 2010). During neural differentiation, 3% O_2 treatment augments the expression of HIF-1α, phosphorylated ERK and p38MAPK. Further, 3% O_2 /hypoxia treatment promoted dopaminergic differentiation of mesenchymal stem cells (MSCs) through upregulation of phosphorylated p38MAPK and subsequent nuclear translocation of HIF-1α in the PD rat model. These changes are followed by promotion of neurosphere formation and DAergic neuronal differentiation, thereby promoting intrastriatal transplantation therapy in PD (Wang *et al.,* 2013). Reduced activity of PHD2 elucidated protection against mitochondrial stress-induced neurotoxicity via HIF-1α expression in dopaminergic neurons and in cultured human-induced pluripotent stem cell-derived neurons (Rajagopalan *et al.,* 2016). Recently, knockdown of Drosophila HIF-1α ortholog Similar (Sima) has been shown to restore neuronal function in Drosophila

model of familial PD without affecting the primary mitochondrial defect, manifesting that mitochondrial retrograde signaling is somewhat accountable for neuronal dysfunction (Cagin *et al.,* 2015). Another study has identified DJ-1 as a mediator of neuronal survival through regulation of the VHL-HIF-1α pathway. Evidence suggests that DJ-1 negatively regulates VHL ubiquitination activity of the α-subunit of HIF-1α by inhibiting HIF-VHL interaction, thereby demonstrating neuroprotection against MPP+ -induced toxicity (Parsanejad *et al.,* 2014). Further, pre-treatment with chronic intermittent hypobaric hypoxia (CIHH) has also been shown to improve ischemia induced cognitive impairment through induction of ERK1/2-CREB-BDNF pathway in anesthetized mice (Wang *et al.,* 2016). Furthermore, ANG administration showed involvement in endogenous protective pathways of motoneurons exposed to hypoxia, thereby suggesting that loss of function rather than loss of expression of ANG is associated with ALS (Sebastià *et al.,* 2009). Mounting evidences advocate that the loss of the protective response triggered by HIF-1/VEGF system under hypoxia causes motor neuron degeneration in ALS. In this case, targeting HIF-1 and/ or its downstream effector VEGF may be a therapeutic approach to postpone ALS associated neuropathology (Correia and Moreira 2010). While in case of HD brain, chemical preconditioning with 3-NP an irreversible inhibitor of succinate dehydrogenase (SDH) has been observed to augment tolerance against experimental hypoxia (Turan *et al.,* 2008). Further, several other suitable approaches still need to be formulated in the near future for efficacious treatment against hypoxia induced neuronal loss.

Owing to scarcity of animals and limitations of sample availability of hypoxic patients the current research scenario has shifted focus on cellular models of hypoxia which are an excellent source of large drug screening and are easy to maintain. The present study uses cell culture model to study the toxic effects of a $CoCl_2$ in cell lines. Moreover, the protective effect of biomolecules/flavanoids against $CoCl_2$ induced toxicity has been studied which will be addressed in Chapter V.

1.8 AIMS AND OBJECTIVES OF PRESENT STUDY

Aim: Elucidating the role of protective biomolecules in hypoxia induced cellular dysfunction.

Objectives of the study: To fulfill the aim of the present work, following objectives were outlined:

(1) To investigate the role of HIF-1α and VEGF in the progression of hypoxia-induced brain damage.

(2) *In silico* analysis of these angiogenic/pro-angiogenic and cell proliferation markers to reveal their interlink between hypoxia and cellular damage.

(3) To analyze the effect of biomolecules on $CoCl_2$ treated SH-SY5Y and HEK-293 cells for exploring molecular mechanistic of hypoxia-induced cellular damage.

(4) To establish therapeutic action of protective bio-molecules in the correction of hypoxia-induced cellular damage.

1.9 PLAN OF WORK

The present work is divided into different sections to achieve the following objectives:

I. Elucidating the potential role of biomolecules to target both HIF-1α and VEGF using various *in silico* tools and techniques.
   - A. Assessing the drug-likeliness of given compounds.
   - B. Studying the pharmacokinetic properties and filtering the compounds on given parameters.
   - C. Carrying out molecular docking studies of selected compounds with angiogenic/pro- angiogenic and cell proliferation markers to assess their regulatory potential.

II. Study the effect of biomolecules in improving toxicity in SH-SY5Y and HEK-293 cells.
   - A. Study the dose-dependent and time-dependent effect of $CoCl_2$ on cell line.
   - B. Study the effect of $CoCl_2$ on cell viability.

C. Check the dose-dependent and time-dependent efficacy of biomolecules against $CoCl_2$ toxicity.

D. Assess the effect of biomolecules on cell viability.

III. Check the protein expression of angiogenic/pro-angiogenic and cell proliferation markers in response to $CoCl_2$ administration and after treatment with biomolecules.

The experimental procedures and methods employed in studying the objectives mentioned above have been addressed in the succeeding chapter.

Chapter II

Experimental Procedures and Materials

CHAPTER II

EXPERIMENTAL PROCEDURESAND MATERIALS

2.1 INTRODUCTION

This chapter outlines the detailed information of various tools and techniques which have been implemented in screening of biomolecules and proteins of interest in studying their role in hypoxia induced organ damage. Also, the experimental procedures employed in sub-culturing cell lines, administering toxin and treating cells with biomolecules/flavonoids have been described. All the experiments which have been performed along with the tools and reagents used are discussed in this chapter.

2.2 *IN SILICO* TECHNIQUES

2.2.1 Data mining

Comprehensive data mining on HIF-1α and VEGF modulators in cellular damage was done with the keywords HIF-1α and VEGF in the NCBI database. Also, extensive literature survey was carried out. The filter criteria were set to HIF-1α and VEGF modulators in the cellular damage under hypoxia accordingly, list of 13 potential compounds was prepared. Out of 13 potential compounds, finally three compounds were filtered and selected for this study.

2.2.2 Retrieval of protein structure and its function recognition

The amino acid sequence of hypoxia inducible factor protein HIF-1α with accession number Q16665.1 of *Homo sapiens* was retrieved from NCBI database and was used for homology search using Basic Local Alignment Search Tool (BLAST). Protein functional elucidation was done using Interproscan server (https://www.ebi.ac.uk/ interpro/search/sequence-search).

2.2.3 Phylogenetic relationship and physico-chemical properties

For multiple sequence analysis Muscle tool (http://www.ebi.ac.uk/Tools/msa/muscle/) was used and phylogenetic tree was constructed using Muscle tool based on NJ (Neighbor joining) plot without distance correction. ProtParam (http://web.expasy.org/protparam/) was used to predict physiochemical properties. The parameters computed by ProtParam included the molecular weight, theoretical PI, aliphatic index and grand average of hydropathicity (GRAVY).

2.2.4 Homology modelling

Homology modeling was used to determine the 3D-structure of HIF-1α and VEGF isoforms. A BLASTP search with default parameters was performed against the Brookhaven Protein Data Bank (PDB) to find suitable templates for homology modeling. Template with PDB ID: 4H6J was retrieved for HIF-1α protein and 1QTY was retrieved for VEGF generated using PDB from PDB. The Protein Structure Prediction Server SWISS MODEL (http://swissmodel.expasy.org/) was used for homology model construction.

2.2.5 Structural visualization and quality assessment

Once the 3D-structure of proteins was generated, structural evaluation was done. Errat server was used to find the accuracy of the structure and visualization of determined structures was performed using UCSF Chimera.

2.2.6 Active site prediction

Castp Server (http://www.sts.bioe.uic.edu/castp/) was used to predict the active sites of HIF-1α protein. Castp could also be used to measure area, circumference of mouth openings of each binding site in solvent and molecular accessible surface. PDB file of protein was uploaded in the server and it showed the ligand binding sites present in protein and the site with maximum surface area and maximum surface volume was selected and all the amino acid residues involved in binding with ligands were retrieved. Furthermore, the active sites of VEGF were predicted using the Pock Drug tool

(http://pockdrug.rpbs.univ-paris-diderot.fr/cgi-bin/index.py?page=home). The PDB structure of VEGF was uploaded and active sites were predicted using f-pocket estimation and setting ligand proximity threshold at 5.5.

2.2.7 Ligand optimization

Reported ligand molecules along with their physical and chemical properties were retrieved from PubChem Compound Database (http://www.pubchem.ncbi.nlm.nih.gov/). Pubchem is a composite database that is backed up by three primary databases, i.e. PCsubstance, PCcompound and PCBioAssay. Pubchem provides biological activity and chemical information of small molecules. PCsubstance contains information about the substances; PCcompound contains information about chemical compounds and PC bioAssay provides information about Bioassays. Initially, three compounds (*Naringenin, Quercetin* and *Sesamol*) were selected. SDF files of Ligands were converted in PDB file with the help of Open Babel tool that could be used for docking study. Visualization of molecular structure of compounds was done using Pymol.

2.2.8 ADMET analysis

The toxicity profiling of compounds was carried out through the online tool Swiss ADME (www.swissadme.ch/index.php). The Swiss ADME tool assessed the compounds on various parameters such as lipophilicity (logP), hydrophilic nature (logS) and BBB permeability. Additionally, two other parameters; Ghose filter and Veber rules were employed for drug-likeliness screening. The qualifying parameters of Ghose filter are (a) molecular weight 160-480 (b) number of atoms 20-70 (c) molar refractivity 40-130 and (d) polar surface area <140 (Ghose et al. 1999). The Veber rules are (a) rotatable bond count <=10 and (b) polar surface area <=140.

2.2.9 Lipinski filter analysis of screened drugs

An online tool Lipinski Filter (http://www.scfbio-iitd.res.in/software/drugdesign/ lipinski.jsp) was used to retrieve the information about drug likeness properties of

biomolecules with the help of Lipinski rule of five (Lipinski et al. 2001). Lipinski rule helps to differentiate drug and non-drug like properties of molecules. It is used to identify the possibility of success or failure due to drug likeliness for molecules fulfilling with two or more of the following rules: (a) Molecular Mass should be less than 500 Dalton, (b) High Lipophilicity (expressed as logP less than 5), (c) Less than 5 hydrogen bond donors, (d) Less than 10 hydrogen bond acceptors and (e) Molar refractivity should be between 40-130.

2.2.10 Preparation of protein and ligand molecules

Preparation of protein involves the addition of polar hydrogen atoms, neutralization of charge and removal of any miscellaneous structures from the protein molecule by Autodock 4.2.1 whereas ligand preparation involves the neutralization of charge.

2.2.11 Molecular docking

Prepared and optimized structures of ligands and protein were ultimately used for molecular docking using Autodock 4.2.1 for predicting the possible protein–ligand interactions and the results that include the understanding of the association that involves H-bonding and hydrophobic interactions were analyzed using LigPlot1.4.5, a program to generate schematic diagrams of protein-ligand interactions.

2.3 *IN VITRO* TECHNIQUES

2.3.1 Chemicals, reagents, drugs and antibodies

High-purity *Naringenin, Quercetin, Sesamol*, DMSO and Penicillin-streptomycin were obtained from Sigma Aldrich (Bangalore, India). Primary antibodies were purchased from Santa Cruz biotech (Santa Cruz, CA) and cell signaling (Danvers, MA). For secondary antibody, HRP conjugated anti-rabbit and anti-mouse polyclonal immunoglobulin was purchased from DAKO (Glostrup, Denmark). All other chemicals used were purchased from Merck, Sigma Aldrich and Thermo Fisher Scientific of highest grade and purity.

2.3.2 Cell lines

The SH-SY5Y is a triple cloned cell line of the parent cell line SK-N-SH. It is of human origin and was sub cloned from a four year old female patient suffering from neuroblastoma (Biedler *et al.,* 1978). The cell line is adrenergic and has also been shown to possess moderate levels of dopamine beta hydroxylase activity (Ross and Biedler, 1985) and thus has served as an excellent *in vitro* model to study PD associated phenomenon. Further, we have also used HEK-293 cell line for our experiment. Human embryonic kidney cells 293, also often recognized as HEK 293, HEK-293, 293 cells, or less specifically as HEK cells, are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. Though popular as a transient expression system, this cell type has also seen wide use in stably transfected forms (i.e. transformed cells) to study a variety of cell-biological questions in neurobiology. The HEK-293 cells have been generally used as an expression device for recombinant proteins. Because of epithelial origin, its biochemical machinery is competent of perform most of the post-translational folding and processing required producing functional, mature protein from a wide spectrum of both mammalian and non-mammalian nucleic acids (Thomas and Smart 2005).

2.3.3 Maintenance of the cell lines

The neuroblastoma cell line SH-SY5Y with an early passage of P4 were grown in Dulbecco's Modified Eagle's Medium/F12 (1:1) growth medium (Gibco; Thermo Fisher Scientific, USA) containing L-Glutamate, 10% heat-inactivated fetal bovine serum (FBS), 1mM sodium pyruvate (1:100 dilution) (Sigma-Aldrich; USA), 1X non-essential amino acids (1:100 dilution) (Sigma-Aldrich; USA), sodium bicarbonate (1:50 dilution) (Sigma-Aldrich; USA) and penicillin-streptomycin (Sigma-Aldrich; USA) in T-25 flasks which were pre-coated in a humidified CO_2 incubator with 5% CO_2 and 95% air at 37^0C. The neuroblasts were regularly monitored under microscope for their morphology, growth and confluency. However the growth medium used for HEK-293 was low glucose DMEM supplemented with fetal calf serum (FCS) and 2mM L-Glutamine.

2.3.4 Sub-culturing and differentiation of cell lines

60-80% confluent cultures were used for sub-culturing; before trypsinization, removed complete growth media and washed the cells with 1xDPBS. Next the cells were incubated with 3ml of 0.05% trypsin-EDTA (for 75cm^2 flask) (0.05%; Gibco; Thermo Fisher Scientific, USA) for 5 minutes at 37^{0}C. The trypsin reaction was stopped by adding growth medium and triturating cells with pipette. Further the cells were washed twice by centrifugation at 1500 rpm for 5 minutes (10ml 1xDPBS each time). The pellet obtained at the bottom was delicately handled and supernatant removed carefully. The pellet was dissolved in residual medium. The cells were seeded (1x10^6 cells/mL) in fresh culture media and incubate at 37^{0}C in 5% CO_2 incubator. Within 3-4 days, cells were found to 60-80% confluent and were used for further experiments. Furthermore, cell density was calculated by using 10µL of this solution mixed with 10µL trypan blue solution (0.4%; Sigma-Aldrich; USA) and counting viable cells in 4 different squares of the hemocytometer. Finally, based on calculated cell density, cells were seeded in new T-25 flasks containing fresh medium and incubated in humidified CO_2 incubator with 5% CO_2 and 95% air at 37^{0}C till they reached 60-80% confluence and were used for further experimentation.

2.3.5 Cobalt (ii) chloride ($CoCl_2$)

The present study uses $CoCl_2$ (Sigma-Aldrich; USA) as a chemical to induce hypoxia in the cell lines via activation of HIF-1α. $CoCl_2$ inhibits PHD enzymes (the oxygen sensors) through replacement of Fe with Co making these enzymes unable to mark HIF-α for degradation and enhance the stability of HIF-1α (Zhang et al. 2014). It is soluble in water (100 mg/ml), yielding a clear and red solution.

2.3.6 $CoCl_2$ treatment for inducing chemical hypoxia

The cells were sub-cultured in 24 well plates as described above and labeled each well. Next, stock solutions of $CoCl_2$ in sterile ddH_2O (Sigma-Aldrich; USA) were prepared for 25mM concentration. $CoCl_2$ treatment was given to cells with each well containing

different concentration in an increasing order ranging from 20 μM, 40μM, 80μM, 100μM, 120μM and 140μM. The cells were incubated at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. Next, the cells were observed and photographed under microscope for changes in morphology and confluence at regular intervals of 12 hours, 24 hours and 48 hours and compared with control and SHAM. The experiment was done to determine dose-dependent and time-dependent effect of $CoCl_2$ on SH-SY5Y cells and was carried out in triplicates.

2.3.7 Biomolecules treatment

After determining the LD_{50} concentration of $CoCl_2$ and the time of incubation required, biomolecules were applied in an increasing dose concentration to control and $CoCl_2$ treated cells. A stock solution of *Naringenin* and *Quercetin* (Sigma-Aldrich; USA) was prepared in a stock solution of 1mM in 0.1% DMSO. *Naringenin* was applied at final concentration of 25μM, 50μM, 75μM and 100μM. Further, *Quercetin* was applied at final concentration of 25μM, 50μM, 75μM and 100μM. The cells were incubated at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. Further, the cells were observed and photographed under microscope for changes in morphology and confluence at regular intervals of 12 hours, 24 hours and 48 hours and compared with control and SHAM. The triplicate experimental observations were used to determine the dose-dependent and time-dependent effect of *Naringenin* and *Quercetin* in $CoCl_2$ induced toxicity in SH-SY5Y cells.

2.3.8 Trypan blue exclusion test

Cell viability assay was done by using trypan blue exclusion test. Both the SH-SY5Y and HEK-293 cells were seeded at a density of $2.0x10^5$ cells/well in 24 well plates and incubated overnight at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. $CoCl_2$ treatment was next given to cells for (12-48) hours. The biomolecule, *Naringenin* (25-100μM) and *Quercetin* (25-100μM) were then added to $CoCl_2$ treated cells and incubated for 48 hours. Viable cells were counted after $CoCl_2$ administration and post-

biomolecules treatment according to the given protocol. The cell suspension (10µL) and 10µL of trypan blue solution (0.4%; Sigma-Aldrich; USA) was mixed and loaded on the chamber underneath glass cover slip on the hemocytometer. The solution was taken up by capillary action and a clean cover slip was carefully placed. The live cells were counted as unstained and dead as blue stained cells in four different squares of hemocytometer using a cell counter under the inverted microscope. Finally, cell viability for each ml was calculated by using the formula: % Cell viability = (number of live cells/total number of cells) x100. Similarly, cell viability was determined for each concentration of $CoCl_2$ and biomolecules at defined time periods in the triplicate experimental sets.

2.3.9 Preparation of cellular protein extract

Cellular protein extracts were prepared by washing both SH-SY5Y and HEK-293 cells in ice cold PBS and scraping into ice cold lysis buffer (50mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 µg/ml PMSF, 0.02% NaN2, 1 µg/ml aprotinin, 1% Triton X-100) containing the protease inhibitor cocktail (Sigma-Aldrich; USA). Lysates were incubated on ice for 20 minutes before centrifugation at 14,000 rpm for 15 minutes at 4°C to remove nuclei and cellular debris. Lysates were analyzed for protein concentration using the Dot-blot method and boiled in sample buffer for SDS-PAGE.

2.3.10 SDS-PAGE and Western blotting

After preparation of cellular protein extract, each protein sample (30 µg) in Lamelli buffer was heated for 10 minutes at 95^{o}C, separated on 10% SDS-PAGE gel and finally, electro-blotted (BioRad; USA) onto a PVDF membrane (GE Healthcare, Piscataway, NJ). Membrane were blocked in 5% (w/v) nonfat dry milk and incubated for 3 hours at room temperature with primary antibody (1:500–1,000 dilution; Santa Cruz; USA) followed by three times washing in 1X TBST buffer. After washing, membranes were incubated in goat anti-mouse IgG or goat anti-rabbit IgG HRP-conjugated secondary antibody (1:5,000 dilutions; Santa Cruz; USA) for one hour. The

autoradiography signals were visualized using ECL advance Western blotting detection kit (RPN2135) on an X-ray film (Thermo Fisher Scientific; USA) after washing the membrane three times.

2.3.11 Ponceau S stain for Western blots

Ponceau S stain was performed to check whether protein was transfer properly on PVDF membrane and were equal in all the lanes. This is a rapid and reversible staining method for locating protein bands on Western blots. Membrane was incubated with Ponceau S for 3-5 minutes, washed with TBST until stain was removed completely and background was clear before blocking the membrane in 5% milk.

2.3.12 Statistical analysis

All data were expressed as mean±SEM. The significance was calculated using one way ANOVA with Tukey's multiple comparison test. $P<0.05$ was considered statistically significant.

After adopting *in silico* tools and techniques outlined in this chapter, we have further performed drug screening for targeting HIF-1α protein to explore their therapeutic potential in cellular damage under hypoxia which has been discussed in the succeeding chapter.

Chapter III

Docking Analysis of Biomolecules to Target Hypoxia Inducible Factor-1α

CHAPTER III

DOCKING ANALYSIS OF BIOMOLECULES TO TARGET HYPOXIA INDUCIBLE FACTOR-1α

3.1 OBJECTIVE

This chapter discusses the comparative study of different biomolecules (*Naringenin, Sesamol* and *Quercetin*) to target HIF-1α. Initially various compounds have been evaluated on drug-likeliness parameters and shortlisted candidates which qualify all the parameters are used further in the present study.

3.2 BACKGROUND

Hypoxia plays a decisive role in controlling many important signaling molecules in the CNS (Li *et al.,* 2010). At the higher altitude, low barometric pressure causes accelerated expression of HIF gene. Hypoxia inhibits prolyl hydroxylation of HIF-1α leading to aggregation of a functional heterodimeric transcription factor (both HIF1 α and HIF1 β subunit) (Keith *et al.,* 2011). HIF-1 is a key transcriptional factor which is amenable for cellular adaption to low oxygen tension. It is a heterodimer comprising of an oxygen-regulated α-subunit and a constitutively expressed β-subunit that regulates a series of genes associated with iron metabolism, angiogenesis, cell-proliferation/survival and glucose metabolism (Correia *et al.,*2013). The activity of HIF-1 is regulated by post-translational modifications on different amino acid residues of its subunits, mainly the α-subunit. It has been reported that under hypoxic insult, the activity of HIF-1 and expression of its associated down-stream genes, such as VEGF and EPO are altered in a range of NDDs including AD, PD and ALS (Ziello *et al.,* 2007). It has also been reported that hypoxia causes reduced synaptic transmission associated with neuronal death by causing neuronal dysfunction (Mukandala *et al.,* 2016). Thus, elucidation of patho-physiological mechanisms caused due to hypoxic insults on the CNS; their therapeutic regulation awaits much importance. Further, experimental and clinical

evidence has revealed that regulating the expression of HIF-1α might improve the cellular and tissue damage in the NDDs. This regulatory role on the expression of HIF-1α can be accompanied by employing different flavonoids/biomolecules. Flavonoids are a class of plant based phenolic compounds with high content in oranges, grapes, lemons, red wine and green tea. The signature properties of flavonoids include antioxidant, anti-inflammatory, anti-allergic, anti-viral, anti-bacterial, anti-cancer, anti-hypertensive, insulin-sensitizing and anti-ischemic. Moreover, flavonoids are well tolerated in human body and display enhanced bioavailability and negligible toxicity in comparison to their synthetic counter-parts. Furthermore, they have been shown to improve upon disease symptoms by modulating various signal transduction pathways in neuronal damage.

Naringenin (5, 7, 4-trihydroxyflavanone) is a flavanone, mainly found in the citrus fruits and tomato. It is known to be act as a multi-functional agent. For example, it acts as a powerful anti-oxidant, anti-depressant, anti-inflammatory and neuroprotective compound (Oh 2016). Similarly, *Sesamol* is the major constituent of sesame seed oil (*Sesamum Indicum*) possessing powerful antioxidant property. Further it acts as chemoprotective, anti-inflammatory, neuroprotective, hepatoprotective and anti-aging biomolecules. Another, well-known biomolecule, *Quercetin* (2-(3,4-dihydroxyphenyl)-3,5, 7-tryhydroxy-4H-chromen-4-one) possess anti-oxidant, anti-inflammatory and in some cases anti-cancerous activities (Zhang and Lokeshwar 2012). These biomolecules are having neuroprotective properties as proposed to be potent therapeutic agent in many diseases, including cognitive impairment associated with neuronal damage. Although, regulating the function of HIF-1α are crucial to be investigated that have protective effects on the pathological ambiances resulting from hypoxic insults in the brain (Sarkar *et al.,* 2012). Therefore, it is pertinent to understand that HIF-1α regulating compounds or which can regulate the abnormal expression of HIF-1α could be a new line of neurotherapeutics in hypoxia mediated neuronal dysfunction.

We performed comprehensive data mining on HIF-1α modulators in brain damage and carried out the study with three compounds (*Naringenin, Sesamol* and *Quercetin*). However, several *in vitro* studies have explored its neuroprotective activities in different neuronal cell cultures and animal models (Angeline *et al.,* 2013; Sarkar *et al.,* 2012). But comparative study of these biomolecules on the expression of HIF-1α by using *in silico* tools has not been explored so far. In present study, we reported the phylogenetic and physico-chemical properties of HIF-1α gene, since we have selected this gene as a target protein for docking study. Further, Homology modeling and 3D structure validation of proteins were performed to establish model accuracy. Additionally, active site prediction and ligand optimization for protein of interest has been done in order to perform molecular docking. Finally, ligand-protein interactions were studied with the targets of interests; HIF-1α through molecular docking studies. Our results have outlined strong potential of selected three biomolecules namely *Naringenin, Sesamol* and *Quercetin* in regulating the altered levels of HIF-1α. These observations may have promising role in targeting hypoxia induced HIF-1α mediated neuronal loss.

3.3 EXPERIMENTAL AND RESULTS

3.3.1 Retrieval of hypoxia inducible factor protein and its functional elucidation

Based on functional domain sequence of well characterized gene/protein, homology search was done using BLAST. We have successfully hunted 5 isoforms **(Table 3.1)** of protein HIF-1α on the basis of families and domains identified from Interproscan results. Interproscan study revealed that all homologues proteins for HIF-1α were belonging to Hypoxia-inducible factor, α- subunit family (IPR021537), Hypoxia-inducible factor-1α family (IPR001321), Myc-type, basic helix-loop-helix domain (IPR011598), PAS domain (IPR000014), PAS fold (IPR013767), PAS fold 3 (IPR013655), HIF-1α, trans-activation domain, C-terminal (IPR014887) and a repeat of PAC motif (IPR001610) respectively **(Figure 3.1)**.

Table 3.1: Hunted HIF-1α related proteins

S.No.	Accession No.	Protein	Score	Identity	E Value
1	NP_001521.1	hypoxia-inducible factor 1-alpha isoform 1	1721	100%	0
2	AKI70676.1	HIF1A	1719	99%	0
3	AAC68568.1	hypoxia-inducible factor 1 alpha subunit	1718	99%	0
4	NP_001230013.1	hypoxia-inducible factor 1-alpha isoform 3	1696	99%	0
5	Q9XTA5.1	Hypoxia-inducible factor 1-alpha	1624	95%	0

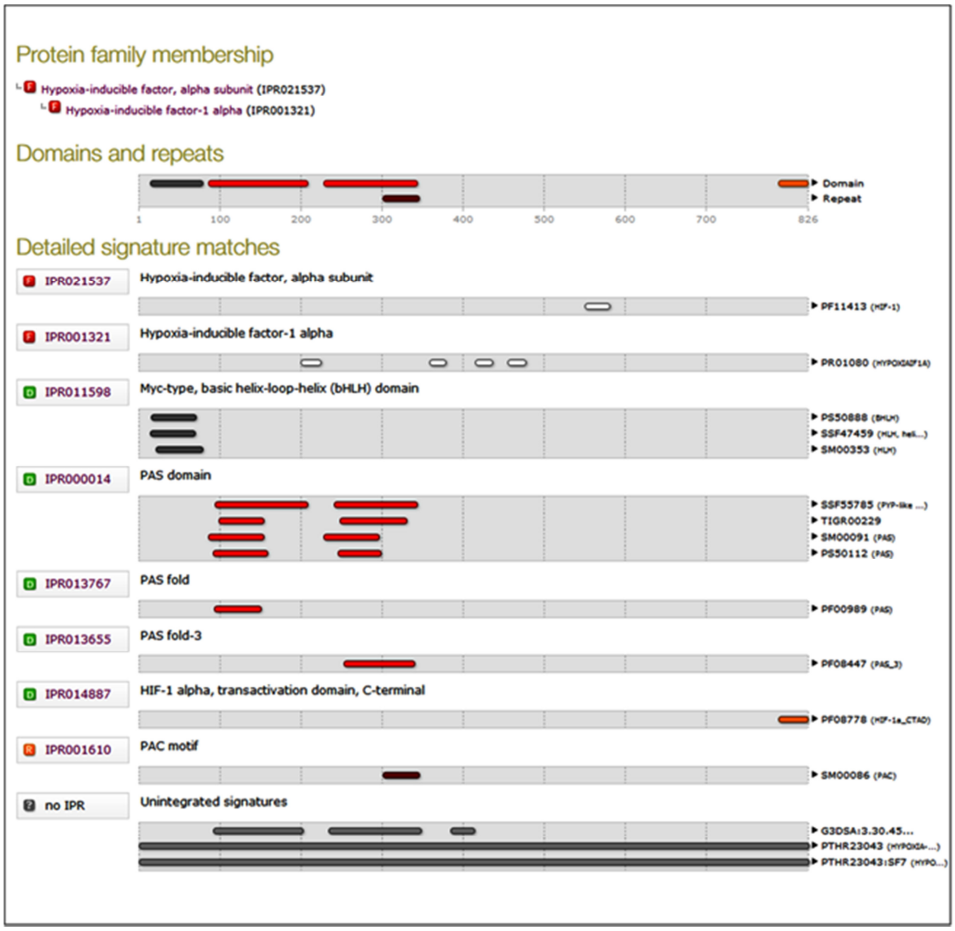


Figure 3.1: Interproscan result for HIF-1-α family and their domain identification

3.3.2 Phylogenetic relationship and physico-chemical properties

For multiple sequence analysis, Muscle tool was used and found that amino acid residues were conserved in most of the isoforms of the protein HIF-1α **(Figure 3.2a)**. Phylogenetic study of HIF-1αhunted proteins revealed that HIF-1α and HIF1A were in same cluster as they share same homology and HIF-1α isoform 1 was in another cluster while HIF-1α isoform 3 and HIF-1α subunit were differ from others **(Figure 3.2b)**. ProtParam showed that Mol. wt. of HIF-1α was 92670.4 Daltons. An isoelectric point for HIF-1αwas 5.17 which indicate that protein was negatively charged. The GRAVY index of -0.573 for HIF-1α is indicative of hydrophilic and soluble protein **(Table 3.2)**.

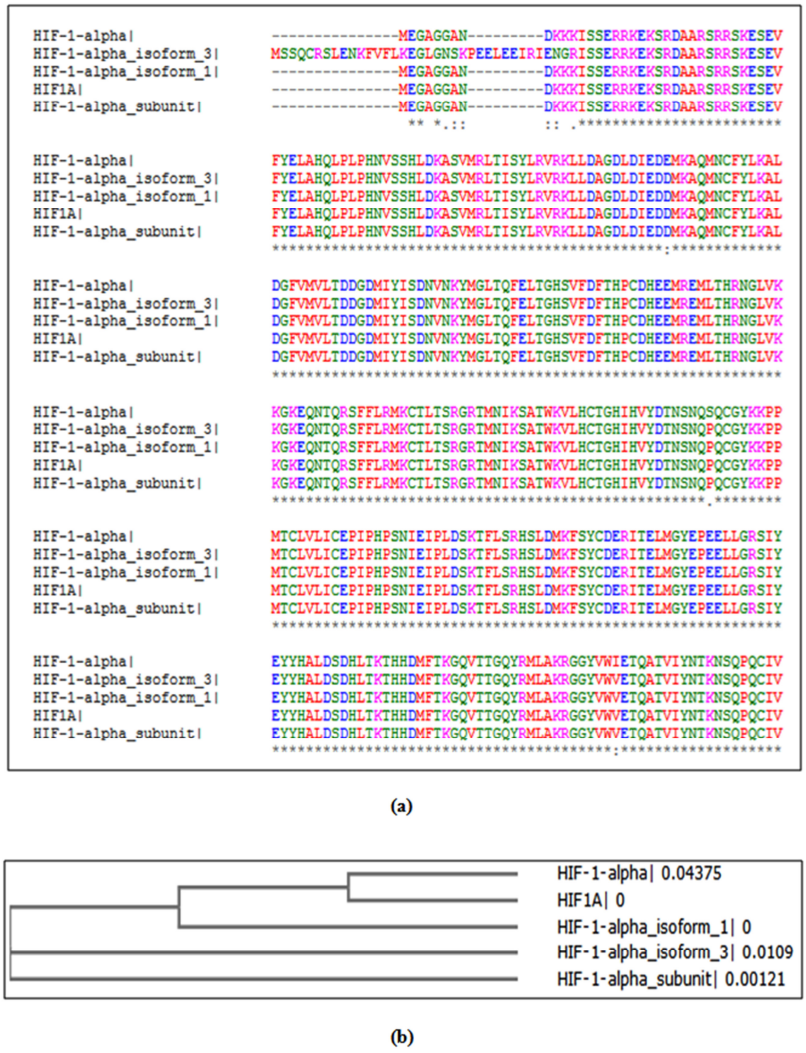


Figure 3.2: (a) Multiple Sequence Alignment and **(b)** phylogenetic Analysis of all HIF-1αisoforms

Table 3.2: Physico-chemical properties of HIF-1α

Properties	**HIF-1α**
Molecular Formula	$C_{4027}H_{6410}N_{1108}O_{1309}S_{43}$
Molecular Weight (Daltons)	92670.4
Theoretical PI	5.17
Aliphatic Index	74.96
Grand Average of Hydropathicity (GRAVY)	-0.573

3.3.3 Homology modelling

Template with PDB ID: 4H6J was retrieved for HIF-1α protein. The Protein Structure Prediction Server SWISS MODEL (http://swissmodel.expasy.org/) was used for homology model construction. Prediction of 3D-structure of proteins provides us precise functional information of how proteins interact and localize in their stable conformation. Homology modelling is a most common structure prediction method in structural genomics and proteomics. The best matching template was selected for the target protein on the basis of sequence homology using PDB Advance Blast. Template is experimentally determined 3D-structure of protein that share sequence similarity with target sequence. Template showed sequence identity of 99.07% for HIF-1α isoforms. 3D-structure of HIF-1α was generated using Swiss Model Server. The Z-score is indicative of overall model quality and is used to check whether the input structure is within the range of scores typically found for native proteins of similar size. Z-score of the template and query model was obtained by SWISS MODEL. Z-score for HIF-1α was -0.81 suggesting a good structure **(Table 3.3)**.

Table 3.3: Swiss Model server result showing template structure used in homology modelling, sequence identity and quality score of the model generated

Gene Name	**Modelled residue range**	**Based on template**	**Sequence identity**	**QMEAN Z-Score**
HIF-1α	139-811	4H6J	99.07%	-0.81

3.3.4 3D-Structure visualization and quality assessment

3D-structure of HIF-1α transcription factor was generated and visualized using UCSF Chimera **(Figure 3.3a)**. Even though there were no steric clashes in the structure generated, it was assessed for geometric and energy aspects. Errat server was used to determine the accuracy of the model. Result of Errat showed 95.694% accurate structure for HIF-1α protein.

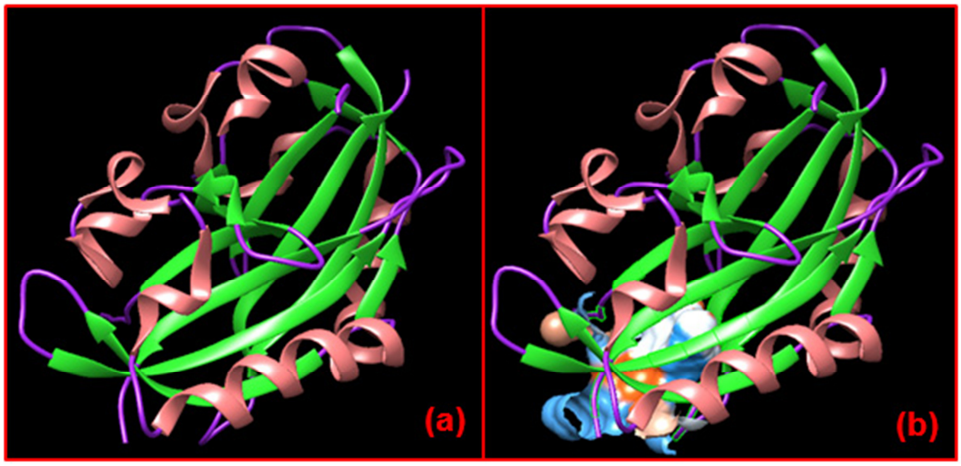


Figure 3.3: (a) 3D-Structure and **(b)** Ligand binding site in HIF-1α

3.3.5 Active site prediction

CastP server was used to predict the ligand binding sites in the generated 3D-structure of HIF-1α. This server calculates the possible active sites from the 3D atomic coordinates of the protein **(Figure 3.3b)**. Among the twenty nine binding sites obtained from CastP for HIF-1α, site 29 was highly conserved within the active site of the protein. The Predicted site 29 consisted 415.4 cubic angstroms site volume out of the 1661.1 cubic Angstroms of protein volume.

3.3.6 Screening for drug-likeness of compounds

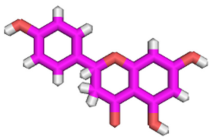
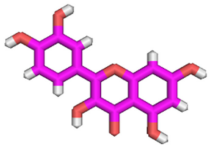
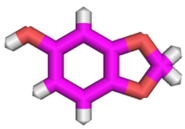
Most of the compounds passed drug-likeness parameters but failed ADMET analysis predictions (**Table 3.4**). Out of thirteen compounds initially screened through literature survey, only three compounds were found to meet the ADMET parameters. These

compounds were *Naringenin, Quercetin* and *Sesamol*, which qualified all the parameters and were used in Physico-chemical properties of natural compounds used for docking study (**Table 3.5**). After structural analysis of HIF-1α and drug screening, we have performed docking study.

Table 3.4: ADMET analysis of selected compounds

Compounds	Physicochemical Properties				Pharmacokinetics		Lipophilicity	Drug-likeness				Water Solubility
	Mol. Weight	H-bond Acceptors	H-bond Donors	Molar Refractivity	GI Absorption	BBB Permeability	XLogP	Lipinski	Ghose	Veber	Bioavailability Score	Log S (EOSL)
Curcumin	368	6	2	102	High	No	3.2	Yes	Yes	Yes	0.55	-3.94
Valproic acid	144	2	1	42	High	No	2.75	Yes	No	Yes	0.56	-2.14
Vitamin B12	1355	20	9	351	Low	No	-3.85	No	No	No	0.17	-4.11
Telmisartan	514	4	1	157	Low	No	-3.85	No	No	Yes	0.56	-7.5
Naringenin	**272**	**5**	**3**	**71**	**High**	**Yes**	**-3.85**	**Yes**	**Yes**	**Yes**	**0.55**	**-3.49**
Deferoxamine	560	9	6	142	Low	No	-3.85	No	No	No	0.17	-0.14
Dabigatran	471	6	4	134	Low	No	-3.85	Yes	No	No	0.55	-3.62
SB 203580	377	4	1	105	High	No	-3.85	Yes	No	Yes	0.55	-4.56
Quercetin	**302**	**7**	**5**	**78**	**High**	**Yes**	**-3.85**	**Yes**	**Yes**	**Yes**	**0.55**	**-3.16**
Mimosine	198	5	3	48	High	No	-3.85	Yes	No	Yes	0.55	1.56
Sesamol	**138**	**3**	**1**	**44**	**High**	**Yes**	**-3.85**	**Yes**	**No**	**Yes**	**0.55**	**-1.92**
Vitamin D	384	1	1	125	Low	No	-3.85	Yes	No	Yes	0.55	-6.84
Orexin-B	2936	40	41	736	Low	No	-3.85	No	No	No	0.17	-1.78

Table 3.5: Physico-chemical properties of natural compounds used for docking study

Characteristics	***Naringenin***	***Quercetin***	***Sesamol***
Molecular weight	272.25278 g/mol	302.2357 g/mol	138.12074 g/mol
Molecular Formula	$C_{15}H_{12}O_5$	$C_{15}H_{10}O_7$	$C_7H_6O_3$
Molecular Structure			
IUPAC Name	5,7-dihydroxy-2-(4-hydroxy phenyl)-2,3-dihydrochromen-4-one	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	1,3-benzodioxol-5-ol
Rotatable Bond Count	1	1	0
Topological Polar Surface Area	$87A^2$	$127A^2$	$38.7A^2$
Heavy Atom Count	20	22	10
Complexity	363	488	126

3.4 DOCKING CALCULATION OF COMPOUNDS WITH HIF-1α

3.4.1 HIF-1α interaction with *Naringenin*

Free energy of binding with *Naringenin* was-8.48 kcal/mol and Est. Inhibition Constant, Ki was found to be 605.40nM. Intermolecular Energy was found to be -9.68 kcal/mol. VdW + Hbond + desolv Energy and Electrostatic Energy was -9.62 kcal/mol and -0.06 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be 9.70 kcal/mol and 1.19 kcal/mol.

3.4.2 HIF-1α interaction with *Quercetin*

Free energy of binding with *Quercetin* was -8.22 kcal/mol and Est. Inhibition Constant, Ki was found to be 945.18 nM. Intermolecular Energy was found to be -10.01 kcal/mol. VdW + Hbond + desolv Energy and Electrostatic Energy was -9.81 kcal/mol and -0.20 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be 9.51 kcal/mol and 1.79 kcal/mol.

3.4.3 HIF-1α interaction with *Sesamol*

Free energy of binding with *Sesamol* was -5.13 kcal/mol and Est. Inhibition Constant, Ki was found to be 174.23 µM. Intermolecular Energy was found to be -5.43 kcal/mol. VdW + Hbond + desolv Energy and Electrostatic Energy was -5.34 kcal/mol and -0.08 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be 0.32 kcal/mol and 0.30 kcal/mol. Docked energy estimation of HIF-1α is shown in (**Table 3.6)** and interaction of HIF-1α with ligands is shown in (**Figure 3.4)**.

Table 3.6: Docking calculation of compounds with HIF-1α

Compound Name	Est. Free Energy of Binding	Est. Binding Constant	Est. Intermolecular Energy	vdW+Hbond+ desolv Energy	Electrostatic Energy	Est. Internal Energy	Torsional Free Energy
Naringenin	-8.48 (kcal/mol)	605.40 nM	-9.68 (kcal/mol)	-9.62 (kcal/mol)	-0.06 (kcal/mol)	+9.70 (kcal/mol)	+1.19 (kcal/mol)
Quercetin	-8.22 (kcal/mol)	945.18 nM	-10.01 (kcal/mol)	-9.81 (kcal/mol)	-0.20 (kcal/mol)	+9.51 (kcal/mol)	+1.79 (kcal/mol)
Sesamol	-5.13 (kcal/mol)	174.23 µM	-5.43 (kcal/mol)	-5.34 (kcal/mol)	-0.08 (kcal/mol)	+0.32 (kcal/mol)	+0.30 (kcal/mol)

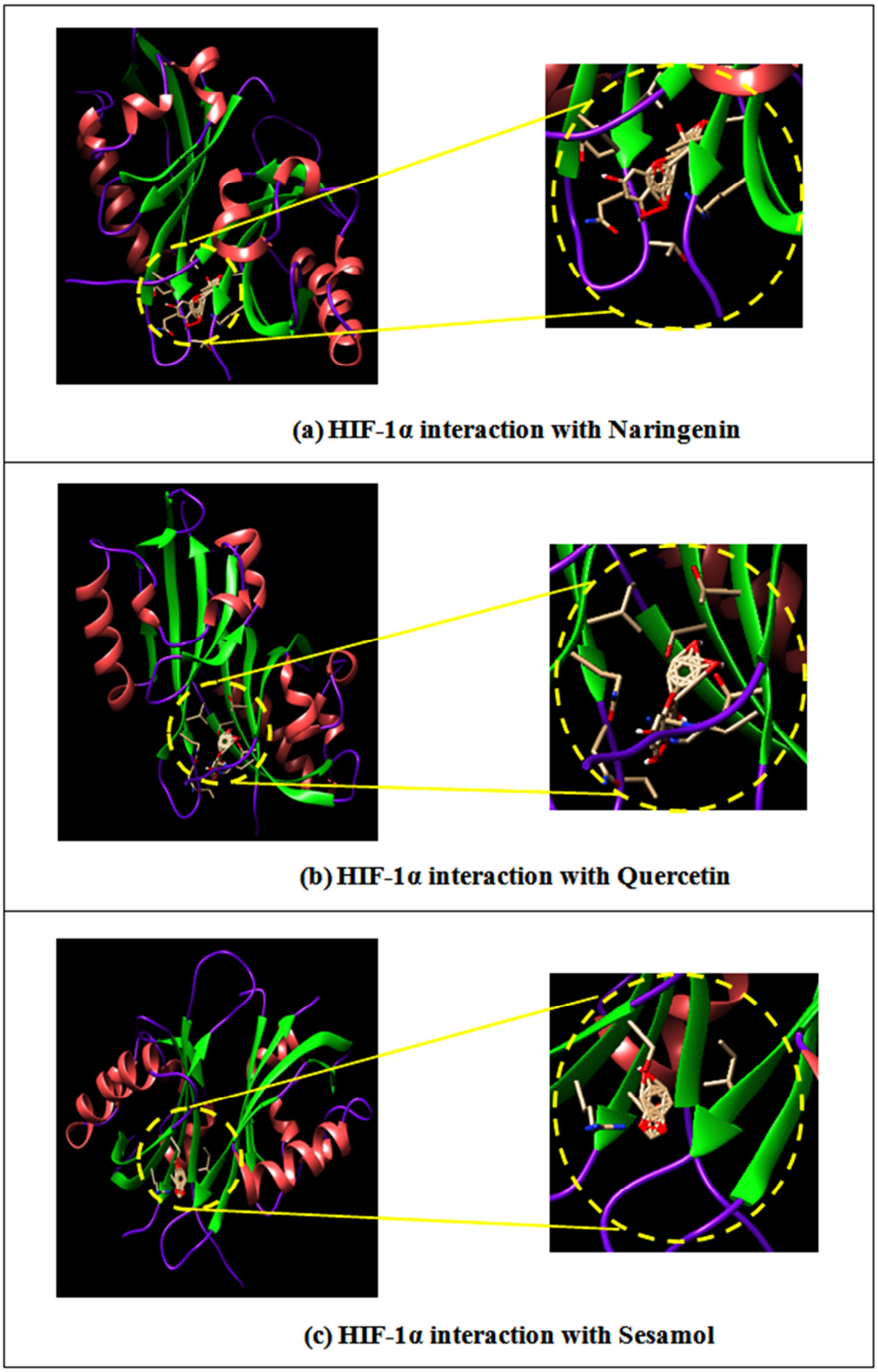


Figure 3.4: Docking study of HIF-1α protein with selected compounds: (a) HIF-1α interaction with *Naringenin*, **(b)** HIF-1α interaction with *Quercetin* and **(c)** HIF-1α with *Sesamol*

3.4.4 Binding site of HIF-1α with selected compounds along with its reported Inhibitory active site

Binding site residues of HIF-1α interacting with *Naringenin, Quercetin* and *Sesamol* were found to be the same as the residues involved in their respective catalytic sites. Interacting residues of HIF-1α with *Naringenin, Quercetin* and *Sesamol* along with their identified catalytic sites have been shown in (**Table 3.7**) and their 2D and 3D pattern of interaction is presented in (**Figure 3.5)**.

Table 3.7: HIF-1α known inhibitory site and selected compounds interacting residues

Compounds	**Interacting residues**
Predicated Active Site	ILE^{324}, TYR^{325}, ASN^{326}, THR^{327}, LYS^{328}, GLN^{333} and CYS^{334} of chain A and CYS^{358}, GLN^{359}, PRO^{360}, ARG^{362}, MET^{426}, ARG^{440}, THR^{441}, SER^{442}, THR^{460}, ASN^{461}, THR^{462}, ASN^{463}, VAL^{464} and LYS^{465} of chain B.
Naringenin	ILE^{324}, ASN^{326}, TYR^{325}, THR^{327} and GLN^{333} residues of chain A and PRO^{360}, ARG^{440}, THR^{441}, SER^{442}, THR^{460}, THR^{462} and VAL^{464} residues of chain B.
Quercetin	ILE^{324}, TYR^{325}, ASN^{326}, THR^{327} and GLN^{333} residues of chain A and PRO^{360}, ARG^{440}, SER^{442}, THR^{460}, ASN^{461} and THR^{462} residues of chain B.
Sesamol	ILE^{324}, TYR^{325} and ASN^{326} residues of chain A and ARG^{440}, THR^{441} SER^{442}, THR^{460} and THR^{462} residues of chain B.

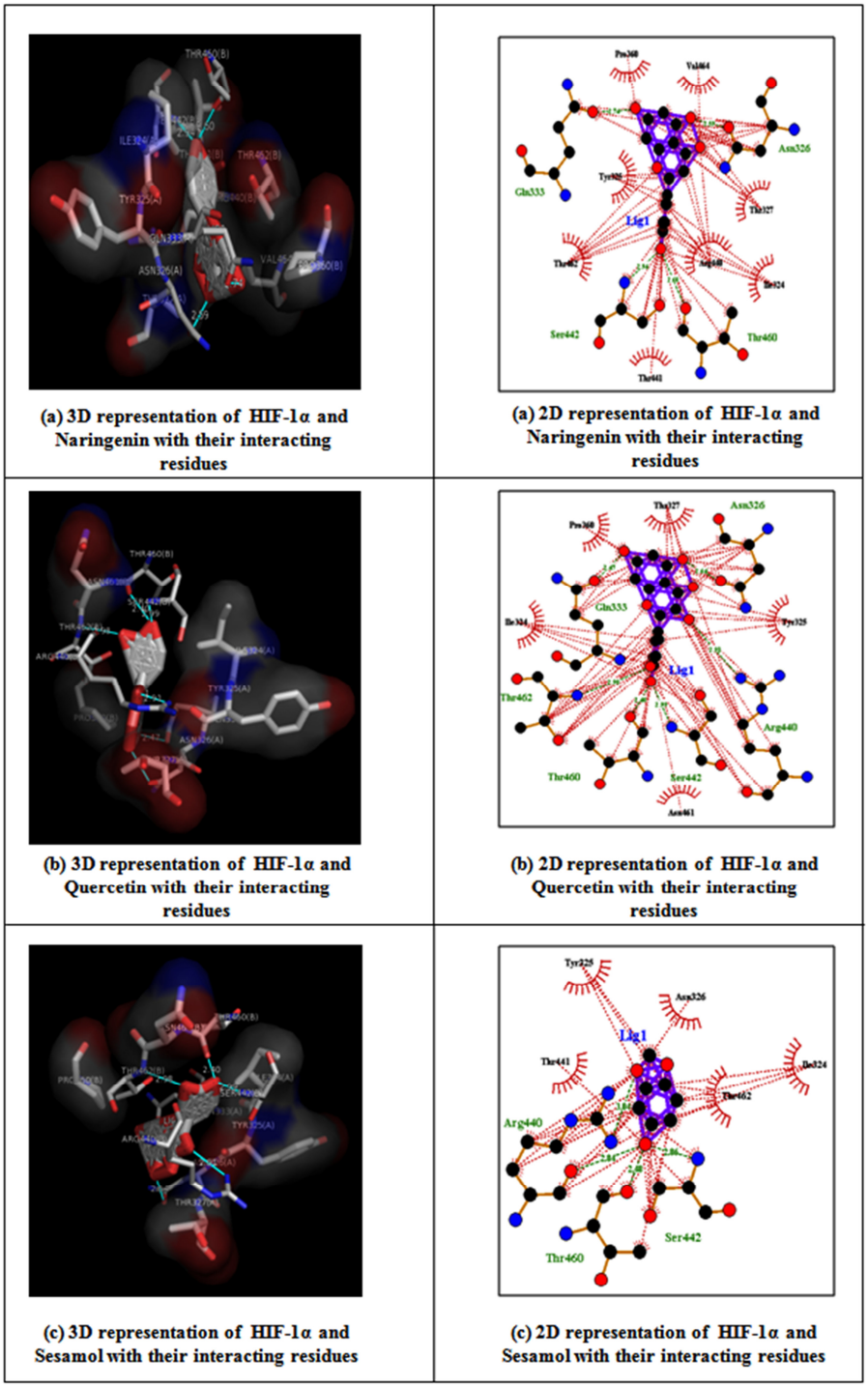


Figure 3.5: Three-dimensional (3D) representation of (a) HIF-1α and *Naringenin* with their interacting residues, **(b)** *Quercetin* with their interacting residues and **(c)** *Sesamol* with their interacting residues. **Two dimensional (2D) representation of (a)** HIF-1α and *Naringenin* with their interacting residues, **(b)** *Quercetin* with their interacting residues and **(c)** *Sesamol* with their interacting residues

3.5 DISCUSSION

Further recent therapeutics advancement in hypoxia mediated aberrations reveals the promising role of natural compounds as potent neuroprotective agents. By this *in silico* investigation, we have successfully hunted 5 unique hits using BLAST (Altschul *et al.,* 1990) based on functional domain sequence and optimized the full length genes of HIF-1α on the basis of families and domains identified from Interproscan results. These isoforms belong to Hypoxia-inducible factor, alpha subunit family (IPR021537), Hypoxia-inducible factor-1 alpha family (IPR001321), Myc-type, basic helix-loop-helix domain (IPR011598), PAS domain (IPR000014), PAS fold (IPR013767), PAS fold 3 (IPR013655), HIF-1 alpha, transactivation domain, C-terminal (IPR014887) and a repeat of PAC motif (IPR001610) and catalyse functions based on its activity to regulate the transcriptional activity in areas of vascularization and angiogenesis, energy metabolism, cell survival and tumour invasion. Further, Phylogenetic study of HIF-1α revealed that HIF-1α and HIF1A were in same cluster as they share same homology and hypoxia-inducible factor 1α isoform 1 was in another cluster while hypoxia-inducible factor 1-alpha isoform 3 and hypoxia-inducible factor 1α subunit were differ from others (Edgar 2004). ProtParam results showed that isoelectric point (pI) of HIF-1α was 5.17 which was less than 7 (pI<7), revealing the acidic nature of protein. This computed pI may be useful for developing buffer system for purification by isoelectric focusing method (Ahmed *et al.,* 2013). The GRAVY index is -0.573 for HIF-1α is indicative of hydrophilic and soluble protein. The more hydrophilic compounds likely have better physicochemical parameters such as the lipophilicity and solubility that are pivotal for favorable enthalpic binding whereas, the highly hydrophobic compounds have been recognized to have problems with bioavailability, solubility and selectivity. Template showed 99.07% sequence identity for HIF-1α protein which is another important property for proper ligand interaction. 3D-structure of HIF-1α was generated by using SWISS MODEL Server (Arnold *et al.,* 2006) and visualized using UCSF Chimera (Yang *et al.,* 2012). Z score for HIF-1α was 0.81 respectively suggesting that input structure is within the range of scores typically found for native proteins of similar size. The Z-score indicates overall model quality and measures the deviation of the total

energy of structure with respect to an energy distribution derived from random conformations (Sharma *et al.,* 2011). Further, result of Errat showed 95.694% accurate structure for HIF-1α protein. Afterwards, active site prediction was performed since it is useful to determine potential sites for ligand binding in molecular docking (Dundas *et al.,* 2006). Among the twenty nine binding sites obtained from CastP Server for HIF-1α, site 29 was highly conserved within all the binding sites of HIF-1α protein. Three compounds (*Naringenin, Quercetin* and *Sesamol*) obtained from different medicinal plants were selected for molecular docking study at *in silico* level.

Initially, we screened the compounds for ADMET and pharmacokinetics analysis. Most drugs fail on poor solubility which we have identified and selected through literature survey. ADMET analysis of all the selected compounds (*Naringenin, Quercetin* and *Sesamol*) revealed that these compounds could act like a drug and have drug like property as these compounds meet the criteria of ADMET parameters. It is evident that *in vivo* bioavailability of an orally administered drug is largely dependent on its aqueous solubility and dissolution in GI fluids. More the water solubility and GI permeability, better the bioavailability. Similarly, lipophilicity of a drug affects various physiological properties such as the rate of metabolism, transport across cell membrane and interaction with binding sites of receptor. Further, drugs intended for CNS should have logP value less than four (Chico *et al.,* 2009). All these three compounds (*Naringenin, Quercetin* and *Sesamol)* showed logP values of -3.85. However, the most important property required of a compound to be a protective agent is the ability to cross BBB. As expected, most compounds failed the BBB permeability parameter. But three biomolecules namely, *Naringenin, Quercetin* and *Sesamol* could cross the BBB and combined with their high GI absorption, least violations of drug likeness and good bioavailability score, were the best candidates for targeting neuronal damage in our study.

Finally, docking study revealed that all three compounds are interacting at the reported active and binding site (Pradeepkiran *et al.,* 2015; Park *et al.,* 2006). Inhibition Constant, Ki of *Naringenin, Quercetin* and *Sesamol* for HIF-1α was found to be 605.40

nM, 945.18 nM and 174.23 μM respectively suggesting that all the selected compounds are effective as HIF-1α modulators. Investigation of active and binding sites within HIF-1α protein gives a better idea for a valuable drug target site and drug interaction with highest affinity. In this result, the most effective compound was found to be *Naringenin* as showing minimum Inhibition Constant, Ki and highest negative free energy of binding with maximum interacting surface area (Sarath and Anjali 2017; Manjula and Maheswari 2017). In the light of the above analysis, we found that modulating the HIF-1α activity could be helpful for curing many disease progressions. Since, altered expression of this important transcription factor directs many abnormalities, including neuronal dysfunction and cancers. Thus, the role of natural compound *Naringenin* with HIF-1α provides a novel remedial approach among all three biomolecules based on docking studies and provide a potential curative biomarker for the treatment of patients suffering from hypoxic injuries.

After structural and docking analysis of HIF-1α protein with selected compounds, we wanted to check the effect of selected compounds on the VEGF activity. Since, impaired expression of VEGF leads to neurotoxicity and cellular damage under hypoxia. Therefore in order to explore the therapeutic potential of selected compounds to target VEGF, structural and docking analysis of VEGF with screened compounds was performed and has been addressed in succeeding chapter.

Chapter IV

Biomolecules Mediated Targeting of Vascular Endothelial Growth Factor in Cellular Dysfunction: An In Silico Approach

CHAPTER IV

BIOMOLECULES MEDIATED TARGETING OF VEGF IN CELLULAR DYSFUNCTION: AN *IN SILICO* APPROACH

4.1 OBJECTIVE

This chapter discusses the comparative study of different biomolecules (*Naringenin, Sesamol* and *Quercetin*) to target VEGF protein. Initially various compounds have been evaluated on drug-likeness parameters and shortlisted candidates which qualify all the parameters are used further in the present study.

4.2 BACKGROUND

Neurodegenerative diseases are pathological conditions that have an insidious onset and chronic progression. Different models have been established to study these diseases in order to understand their underlying mechanisms and to investigate new therapeutic strategies (Schlachetzki *et al.,* 2013). Several downstream signaling molecules are reported to trigger under hypoxia. VEGF is one of them, which is responsible for the formation of new blood vessels (angiogenesis) and lead to the supply of nutrients and oxygen for normal homeostasis (Hohman *et al.,* 2015). Moreover, the crucial role of VEGF in the brain is not restricted only to controlling vessel growth: but it has direct effects on different types of neural cells including NSCs. Conversely, altered expression of this molecule has been implicated in virtually every type of angiogenic disorder, including those associated with cancer, ischemia and inflammation (Storkebaum and Carmeliet 2004). Moreover, studies have also revealed the pathological implication of VEGF in the progression of NDDs including, AD, HD and ALS. Recently genetic studies have revealed that reduced VEGF levels cause neurodegeneration through impairing neural tissue perfusion (Lange *et al.,* 2016). In case of diabetic nephropathy patients, the VEGF levels were

found to be increased and blocking VEGF or VEGF receptor ameliorated diabetic nephropathy in animal models. Increased plasma VEGF expressions have also been observed in CKD patients. Additionally, lethal effects of VEGF have been reported in atherosclerosis and sepsis, which are common hurdle in CKD patients (Doi *et al.,* 2010). Therefore, implementation of different biomolecules may helpful in regulating the altered levels of VEGF in cells. The growing evidence for an etiologic role of VEGF in neurodegeneration provides an underlying principle for considering the therapeutic potential of VEGF for neuronal damage, which are mostly not curable. In this framework we have introduced different biomolecules (*Naringenin, Quercetin* and *Sesamol*) for targeting VEGF. These biomolecules are having chemoprotective, anti-inflammatory, neuroprotective and anti-aging property. For this purpose, we have performed *in silico* based structural and functional analysis of these molecules for revealing its therapeutic importance against neuronal loss via modulating the impaired expression of VEGF. Further to carry out our work we employed various *in silico* tools and techniques, such as drug-likeness parameters namely Lipinski filter analysis, Pock Drug server for active site prediction, AutoDock 4.2.1 and LigPlot1.4.5 for molecular docking studies. The present study outlines the novel potential of biomolecules in regulating VEGF activity for the treatment of different abnormalities associated with impaired VEGF under hypoxia.

4.3 EXPERIMENTAL AND RESULTS

4.3.1 3D-structure visualization and quality assessment

Template with (PDB ID: 1QTY) was retrieved for VEGF and generated from PDB. 3D-structure of VEGF was generated and visualized using UCSF Chimera **(Figure 4.1a and 4.1b)**. Even though there were no steric clashes in the structure generated, it was assessed for geometric and energy aspects. Errat server was used to determine the accuracy of the model. Result of Errat showed 95.694% accurate structure for VEGF.

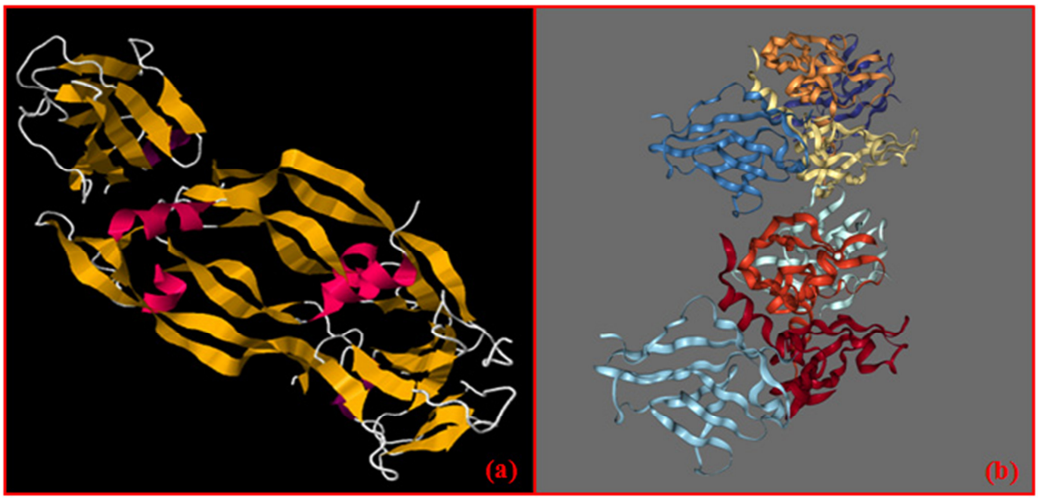


Figure 4.1: (a) 3D-Structure and **(b)** Ligand binding site in VEGF protein

4.3.2 Active site prediction

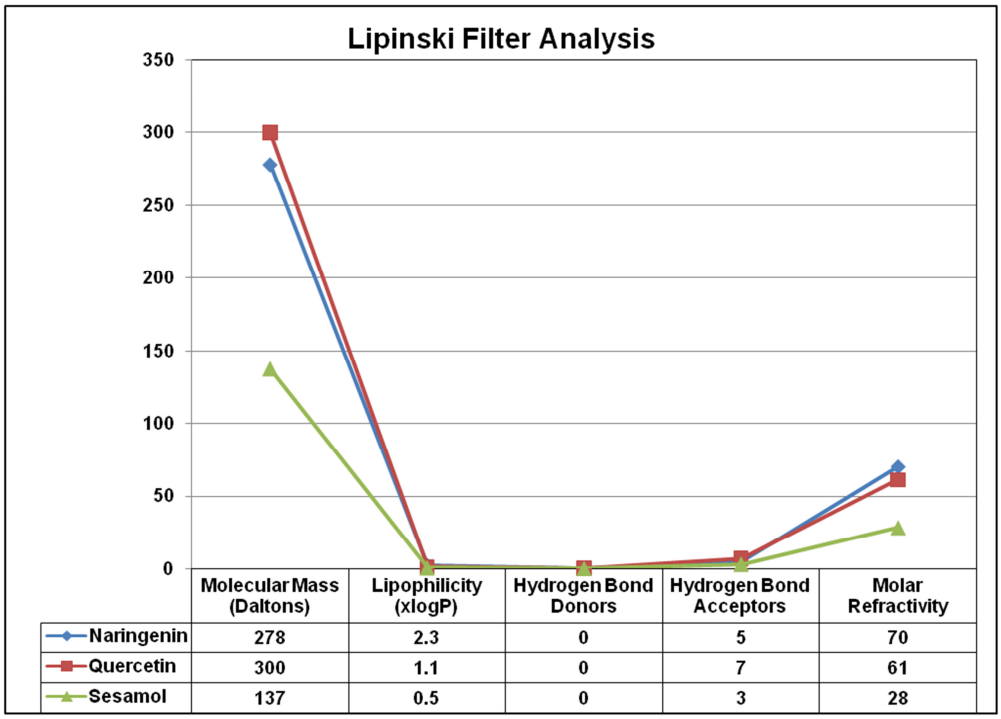
Out of top ten pockets, VEGF had best pocket at P4 with a drugability score of 0.95 and 0.01standard deviation **(Table 4.1)**. The volume of given pocket was 1338.57 cubic angstroms and 21 residues were involved in interaction at this site.

Table 4.1: Active sites of VEGF

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	2205.45	-0.58	0.67	0.2	0.0	30.0	**0.63**	**0.09**
P 1	2500.5	-0.67	0.66	0.19	0.0	32.0	**0.59**	**0.08**
P 16	452.91	0.83	0.43	0.0	0.0	14.0	**0.93**	**0.02**
P 18	744.97	0.56	0.5	0.14	0.0	14.0	**0.94**	**0.01**
P 19	455.66	0.83	0.43	0.0	0.0	14.0	**0.93**	**0.02**
P 2	1622.2	-0.86	0.68	0.24	0.0	25.0	0.49	0.12
P 3	1819.24	-0.87	0.69	0.21	0.03	29.0	**0.53**	**0.03**
P 4	1338.57	0.34	0.62	0.19	0.04	21.0	**0.95**	**0.01**
P 5	1159.82	-0.3	0.67	0.24	0.04	21.0	**0.83**	**0.02**
P 6	1368.17	-1.2	0.75	0.06	0.0	16.0	0.14	0.02

4.3.3 Lipinski filter analysis of screened drugs

Further, the screening of ligand molecules was done on the basis of Lipinski's rule of five. Lipinski filter analysis revealed that all the compounds selected possessed drug likeness and can be used for docking purposes **(Figure 4.2)**.



	Molecular Mass (Daltons)	Lipophilicity (xlogP)	Hydrogen Bond Donors	Hydrogen Bond Acceptors	Molar Refractivity
Naringenin	278	2.3	0	5	70
Quercetin	300	1.1	0	7	61
Sesamol	137	0.5	0	3	28

Figure 4.2: Differentiation of drugs on the basis of Lipinski Rule of five by Lipinski Filter

4.4 MOLECULAR DOCKING OF VEGF WITH BIOMOLECULES

Biomolecules bound to VEGF at P4 pocket and same residues as predicted were involved in the interaction.

4.4.1 VEGF interaction with *Naringenin*

The estimated free energy of binding for VEGF and *Naringenin* was-7.56 kcal/mol and Est. Intermolecular Energy was found to be -9.05 kcal/mol. VdW + Hbond + desolv Energy and Electrostatic Energy was -9.03 kcal/mol and -0.02 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be +9.69 kcal/mol and +1.19 kcal/mol.

4.4.2 VEGF interaction with *Quercetin*

Similarly, the estimated free energy of binding for VEGF and *Quercetin* was-7.10 kcal/mol and Est. Intermolecular Energy was found to be -8.89 kcal/mol. VdW + Hbond + desolv Energy and Electrostatic Energy was -8.73kcal/mol and -0.16 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be +9.49 kcal/mol and +1.79 kcal/mol.

4.4.3 VEGF interaction with *Sesamol*

Likewise, the estimated free energy of binding for VEGF and *Sesamol* was-5.09 kcal/mol and Est. Intermolecular Energy was found to be -5.39 kcal/mol. VdW + Hbond + desolv Energy and Electrostatic Energy was -5.33 kcal/mol and -0.06 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be +0.33 kcal/mol and +0.30 kcal/mol.

Molecular docking pattern of VEGF with screened molecules (*Naringenin, Quercetin* and *Sesamol*) have been identified and depicted in (**Figure 4.3** (**a, b & c**)). On the basis of docking analysis, interacting compounds with minimum binding constant and highest negative free energy of binding are most effective. Docking calculation of VEGF with these molecules has been presented in (**Table 4.2**).

Table 4.2: Docking calculation of compounds with VEGF

Compound Name	Est. Free Energy of Binding	Est. Binding Constant	Est. Intermolecular Energy	vdW+Hbond+ desolv Energy	Electrostatic Energy	Est. Internal Energy	Torsional Free Energy
Naringenin	-7.56 (kcal/mol)	1.74 µM	-9.05 (kcal/mol)	-9.03 (kcal/mol)	-0.02 (kcal/mol)	+9.69 (kcal/mol)	+1.19 (kcal/mol)
Quercetin	-7.10 (kcal/mol)	6.21 µM	-8.89 (kcal/mol)	-8.73 (kcal/mol)	-0.16 (kcal/mol)	+9.49 (kcal/mol)	+1.79 (kcal/mol)
Sesamol	-5.09 (kcal/mol)	186.16 µM	-5.39 (kcal/mol)	-5.33 (kcal/mol)	-0.06 (kcal/mol)	+0.33 (kcal/mol)	+0.30 (kcal/mol)

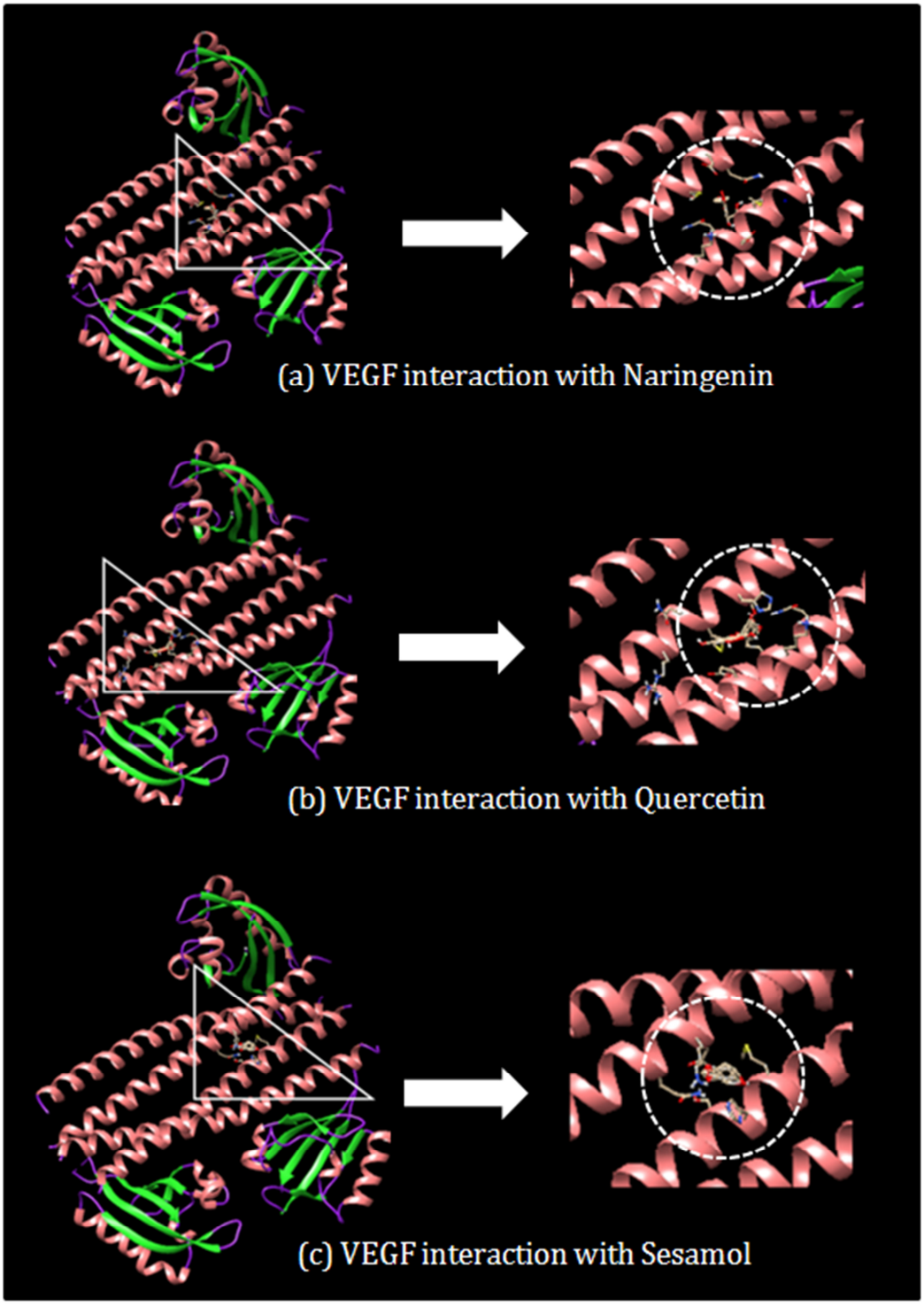


Figure 4.3: Docking study of VEGF protein with selected compounds: (a) VEGF interaction with *Naringenin*, **(b)** VEGF interaction with *Quercetin* and **(c)** VEGF with *Sesamol*

4.4.4 Binding site of VEGF with selected compounds along with its reported Inhibitory active site

Binding site residues of VEGF interacting with *Naringenin, Quercetin* and *Sesamol* were found to be the same as the residues involved in their respective catalytic sites. Interacting residues of VEGF with *Naringenin, Quercetin* and *Sesamol* along with their identified catalytic sites have been show in (**Table 4.3**) and their three-dimensional (3D) pattern of interaction with **(a)** VEGF and *Naringenin* with their interacting residues, **(c)** *Quercetin* with their interacting residues, **(e)** *Sesamol* with their interacting residues. Two dimensional (2D) pattern of interaction with **(b)** VEGF and *Naringenin* with their interacting residues, **(d)** *Quercetin* with their interacting residues, **(f)** *Sesamol* with their interacting residues and also presented in **Figure 4.4**.

Table 4.3: VEGF known inhibitory site and selected compounds interacting residues

Compounds	**Interacting residues**
Predicated Active Site	PHE^{375}, VAL^{376}, ASP^{377}, HIS^{378}, ARG^{379}, VAL^{381}, ALA^{382}, GLY^{385}, GLN^{387}, PRO^{388}, GLN^{389}, GLU^{390}, LEU^{392}, LYS^{432} and ASN^{433} of chain A. GLN^{434}, MET^{605}, THR^{606}, GLU^{608}, GLN^{609}, LYS^{610}, LYS^{612}, GLU^{613}, and GLU^{616} of chain B. MET^{598}, HIS^{601}, SER^{602}, MET^{605}, THR^{606}, LEU^{607}, GLU^{608}, GNL^{609}, THR^{611}, LYS^{612}, GLU^{613}, ILE^{614}, ASP^{615} and GLU^{616} of chain C. ARG^{594}, MET^{598}, HIS^{601}, SER^{602}, MET^{605}, THR^{606}, GLN^{609}, LYS^{612} and GLU^{613} of chain F. SER^{241}, LYS^{242}, ASP^{259}, ARG^{260}, THR^{262}, GLU^{263}, LEU^{264}, ILE^{265}, GLY^{266}, HIS^{268}, PRO^{269}, GLU^{270}, ALA^{311}, LYS^{312}, HIS^{313}, GLY^{314}, GLY^{315}, TYR^{316}, VAL^{317}, TRP^{318}, VAL^{343} and LEU^{344} of Chain G.
Naringenin	MET^{605}, THR^{606}, GLU^{608} and GLN^{609} residues of chain C and MET^{605}, THR^{606} and GLN^{609} residues of chain F.
Quercetin	GLN^{609}, LYS^{612}, GLU^{613} and GLU^{616} residues of chain C and ARG594, MET598, HIS601 and SER602 residues of chain F.
Sesamol	MET598, HIS601 and SER602 residues of chain C and GLN609, LYS612 and GLU613 residues of chain F.

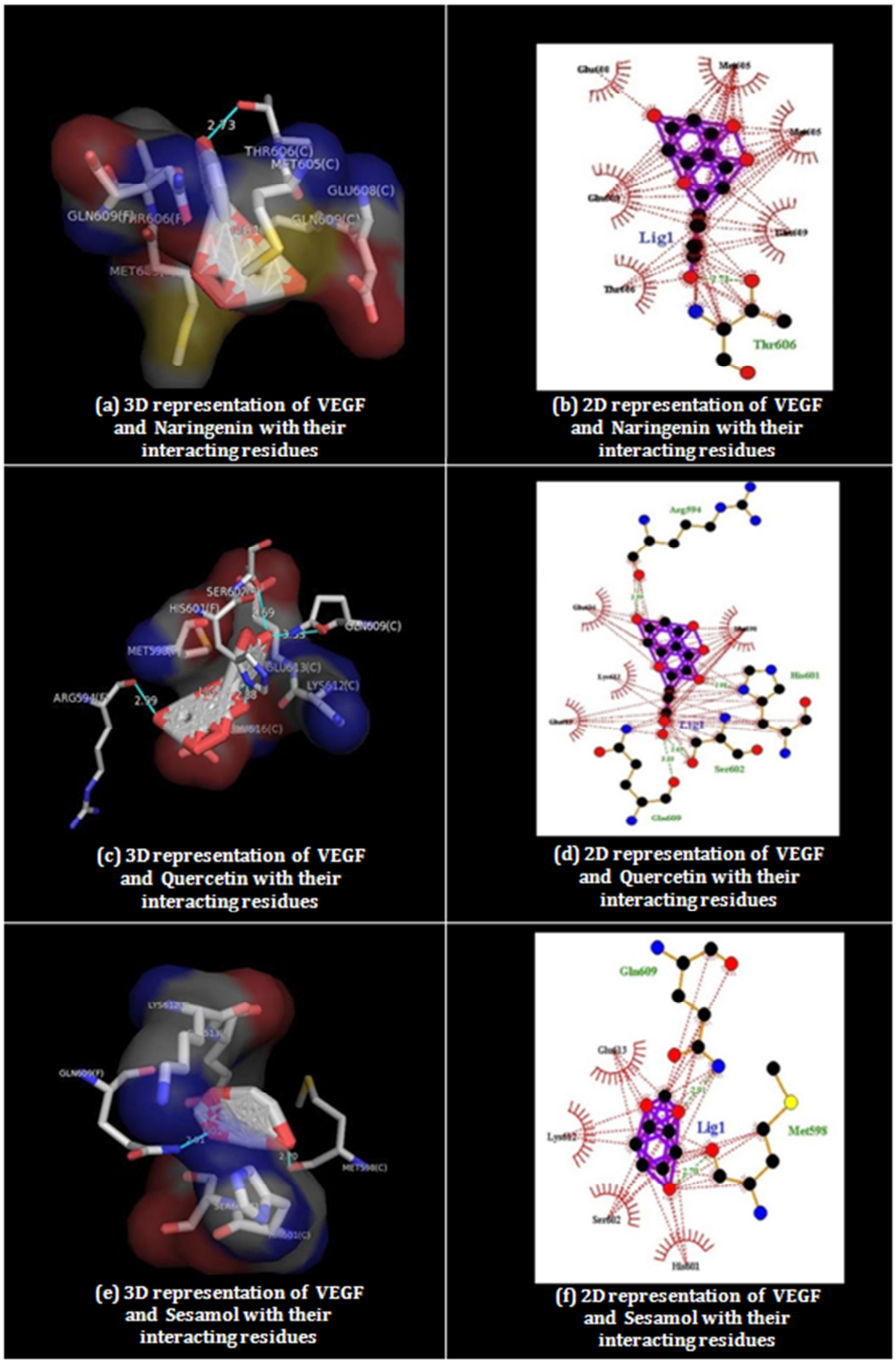


Figure 4.4:Three-dimensional (3D) presentation of (a) VEGF and *Naringenin* with their interacting residues, **(c)** *Quercetin* with their interacting residues and **(e)** *Sesamol* with their interacting residues. **Two dimensional (2D) representation of (b)** VEGF and *Naringenin* with their interacting residues, **(d)** *Quercetin* with their interacting residues and **(f)** *Sesamol* with their interacting residues

4.5 DISCUSSION

Neurodegeneration is the gradual loss of structure and function of neurons (Schneider *et al.,* 2013). Despite the knowledge of various factors which contribute in the occurrence and progression of NDDs, the exact cause and cure remains elusive. Abnormal expression of VEGF protein in terminally differentiated neurons is a recently known phenomenon which has been shown to drive neurodegeneration followed by apoptosis (Wyss-Coray and Rogers 2012). Free radical injury of microvessels under hypoxia causes Neuroinflammation and oligaemia which thereafter leads to Aβ accumulation through vascular damage and the activation of pro-angiogenic factors including, HIF-1α and VEGF-1(Vagnucci and Li 2003). Further, invading macrophages and monocytes also causes neuronal damage via activation of VEGF-1(Dalton *et al.,* 2014). Similarly, low VEGF levels impair spinal cord perfusion and cause chronic ischemia of motoneurons, but also deprive these cells of vital VEGF-dependent survival and neuroprotective signals. Both phenomena result in progressive degeneration of motoneurons, associated with muscle weakness, paralysis and death (Pandey and Rizvi 2009). Further, plasma VEGF levels in diabetic nephropathy patients are also reported to be increased and blocking VEGF or VEGF receptor ameliorated diabetic nephropathy in animal models. Increased plasma VEGF expressions have also been observed in CKD patients. Additionally, lethal effects of VEGF have been reported in atherosclerosis and sepsis, which are common hurdle in CKD patients (Doi *et al.,* 2010). Thus, it seems imperative to design therapeutic strategies aimed at attenuating the altered level of VEGF to inhibit the cascade of cellular damage.

Flavonoids have been advocated to exert human health benefits by anti-oxidant and anti-inflammatory mechanisms (Raza *et al.,* 2013). *Naringenin* reportedly prevent oxidative stress and NF-κB-mediated inflammatory brain damage in the rat model of focal cerebral injury. Further, prophylactic treatment with *Naringenin* ameliorated functional outcomes and abrogated the ischemic brain injury by suppressing NF-κB-mediated Neuroinflammation (Haleagrahara *et al.,* 2011). Similarly, a significant raise in neuronal survivability was observed with *Quercetin* treatment in rats administered 6-

OHDA. Both *Naringenin* and *Quercetin* also reversed the effect of hypobaric hypoxia and elicit neuroprotective response by reducing VEGF level in the murine model (Sarkar *et al.,* 2012). Further, *Sesamol* pre-treatment restored oxidative defense possibly by its free radical scavenging lighted the neuroprotective effect of *Sesamol* against 3-NP- induced neuronal damage (Kumar *et al.,* 2010). Taken together, all these data provide convincing evidence of using VEGF interaction with such as *Naringenin* and *Quercetin* in attenuating the level of VEGF and in turn, inhibit the hypoxia induced cascade of neuronal death.

Lipinski Filter Analysis of all the compounds revealed that these compounds could act like a drug and have drug like property as these compounds meet the criteria of Lipinski Rule of five (Lipinski 2004). Moreover, these biomolecules also met the all criteria of ADMET parameters which we have discussed in previous chapter **(chapter 3)**. Finally, molecular docking studies indicated that all these compounds can bind to and modulate the level of VEGF and possibly, halt or inhibit toxic proteins induced cellular damage. It is also advocated that all the selected compounds for docking study are binding at the reported active site. Further, their binding atomic coordination were compared with the template complex and observed that docked drug coordination was found to be similar with the known coordination. Amino acid residues of VEGF involved in interaction with *Naringenin, Quercetin* and *Sesamol* were found to be the same as the residues involved in binding with earlier used inhibitors. These observations clearly indicate that we can efficiently determine active site coordinates to investigate the effect of inhibitors on the functional active site of protein. The results of our study provide novel potential of biomolecules such as *Naringenin, Quercetin* and *Sesamol* in regulating VEGF expression in the brain, which has wider implications in the progression as well as protection against NDDs. Moreover, out of these three biomolecules *Naringenin* is showing better interaction with VEGF based on their minimum binding constant and highest negative free energy (Maida *et al.,* 2017; Rigoberto *et al.,* 2017; Manjula and Maheswari 2017). These findings can be further validated through *in vitro* and *in vivo* studies in cellular damage. Overall, although further work is required, these studies

advocate the pivotal role of VEGF in neuronal damage and provide adequate grounds for estimating the potential therapeutic effectiveness of VEGF in their management.

After structural and docking analysis of VEGF protein with selected compounds, we wanted to check or validate our *in silico* results with the help of *in vitro* study. For that we have performed *in vitro* experiments by inducing hypoxic stress into the cell lines with the help of chemical inducer. And subsequently checked the effect of screened compounds in toxin treated cells whether these compounds are protective or not for cellular dysfunction under hypoxia. The *in vitro* experimentations for validation of our *in silico* data have been addressed in succeeding chapter.

Chapter V

Protective Effect of Biomolecules in Cobalt (II) Chloride Induced Toxicity in Cell Lines

CHAPTER V

PROTECTIVE EFFECT OF BIOMOLECULES IN COBALT (II) CHLORIDE INDUCED TOXISITY IN CELL LINES

5.1 INTRODUCTION

The previous chapters discussed the biomolecules (*Naringenin, Quercetin* and *Sesamol*) mediated targeting of angiogenic markers such as HIF-1α and VEGF in alleviating toxic proteins mediated cellular dysfunction. Thus, in an attempt to validate the virtual screening results, we carried out various studies to check the efficacy of these biomolecules in ameliorating cellular death in SH-SY5Y and HEK-293 cell line. Also, changes in cell viability subsequent to the effect of toxin and upon biomolecules treatment have been assessed. Moreover, the expression level of various angiogenic markers/cell proliferation markers subsequent to $CoCl_2$ administration and after biomolecules treatment has been described.

5.2 DOSE-DEPENDENT AND TIME-DEPENDENT TOXICITY ASSAY OF $CoCl_2$

A stock of $CoCl_2$ in dH_2O was prepared and from this various gradients of $CoCl_2$ were prepared in increasing concentration of 20μM, 40μM, 80μM, 100μM, 120μM and 140μM. The effect of each of these doses on SH-SY5Y cells was studied in a time period of 12 hours, 24 hours and 48 hours. The results obtained were evaluated through changes in cell morphology, cell viability assay, protein profiling or western blot and finally, statistical calculations and are summarized in succeeding sections.

5.2.1 $CoCl_2$ exerts dose-dependent and time-dependent toxicity in SH-SY5Y cells

The results obtained in present study clearly show that $CoCl_2$ induces toxicity in SH-SY5Y cells in a dose-dependent and time-dependent manner. The change in cell

morphology upon $CoCl_2$ administration in comparison to control cells is clearly evident in **Figure 5.1**.

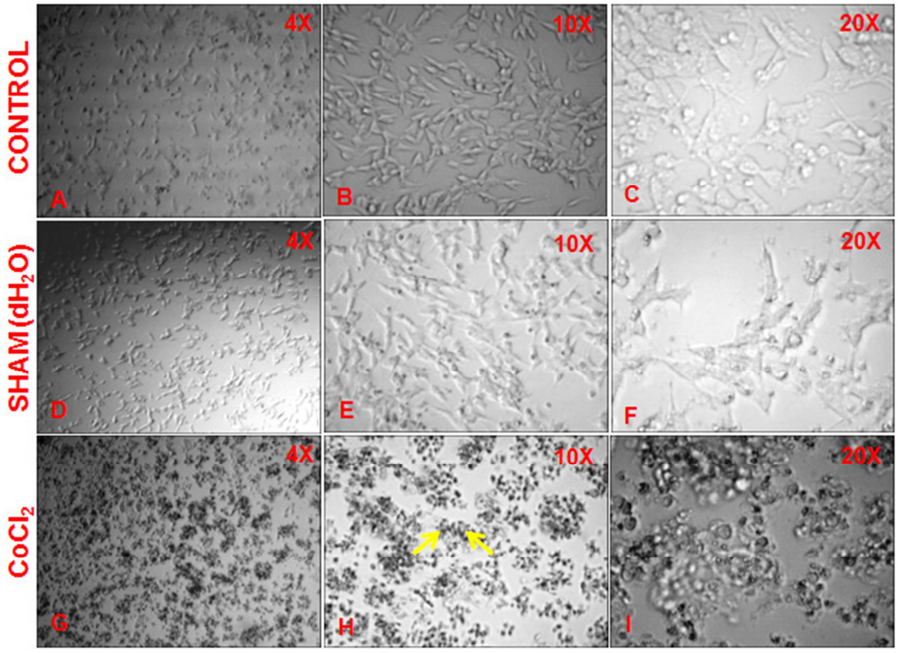


Figure 5.1: $CoCl_2$ changes cell morphology (**G-I**) compared to both control (**A-C**) and SHAM (**D-F**)

5.2.2 $CoCl_2$ exerts mild toxicity at 20µM-80µM

The SH-SY5Y neuroblastoma cells exhibited nearly normal morphology when compared with control and SHAM post 20µM-80µM $CoCl_2$ administration after 24-48 hours (**Figure 5.2**). Most of the cells were alive at these concentrations even after 24 hours. The viability was only slightly decreased at 80µM in comparison to 20µM concentration (**Figure 5.2**).

5.2.3 $CoCl_2$ exerts moderate toxicity at 100µM-120µM

The 100µM concentration of $CoCl_2$ exerted some toxicity after 12 hours. Further, although cells were mostly alive, toxicity at this concentration was visibly apparent after 24 hours. After 24 hours, the confluence of cells was markedly decreased though; the morphology of cells was also abnormal compare to normal cell morphology (**Figure**

5.2). However, the toxicity of 120µM $CoCl_2$ on SH-SY5Y cells was very apparent after 12 hours as cell morphology was visibly distorted. As expected, the morphology degraded after 24 hours and clumps of morphologically abnormal cells were seen after 48 hours of treatment (**Figure 5.2**).

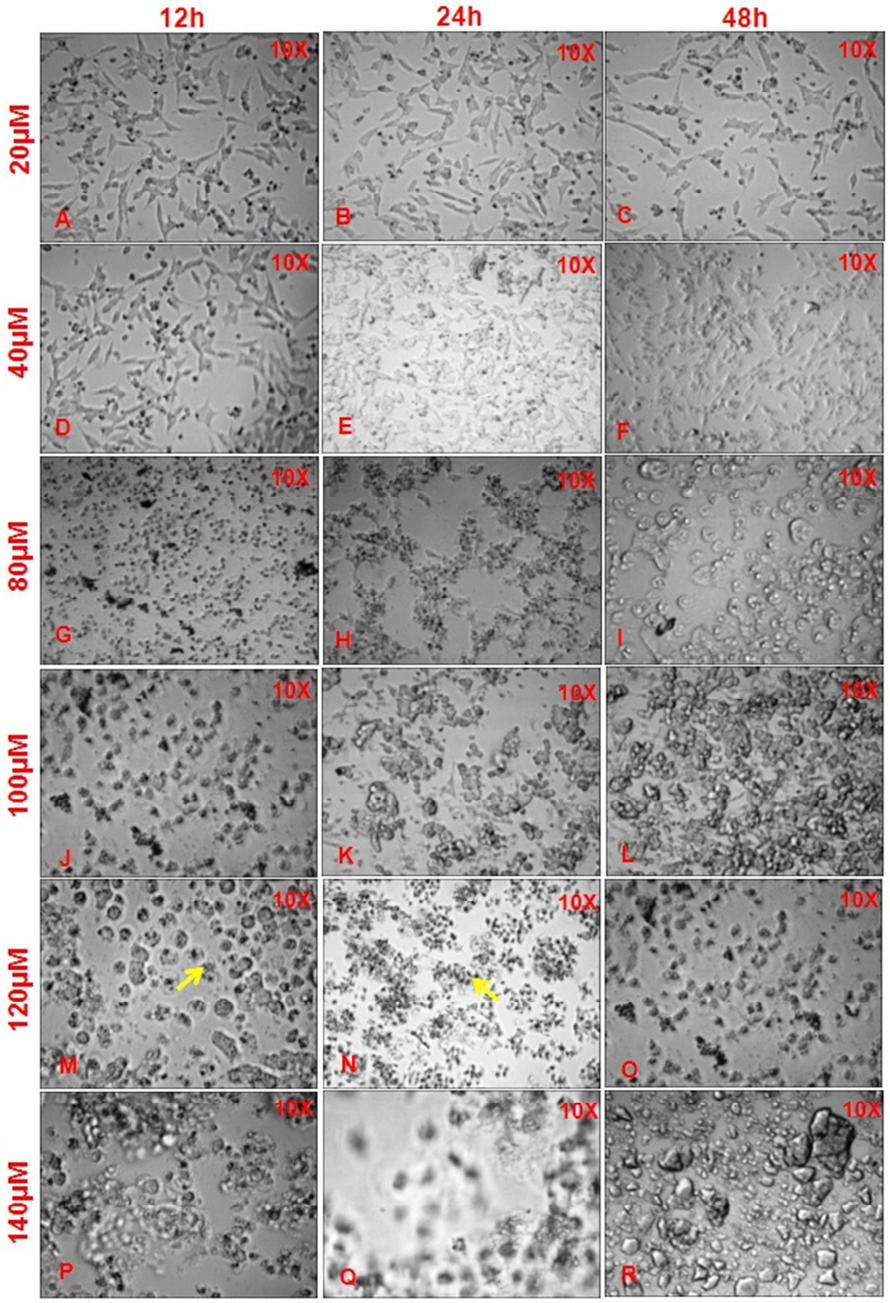


Figure 5.2: $CoCl_2$ exerts mild toxicity at 20µM-80µM (**A-I**). However, moderate toxicity seen at $CoCl_2$ doses 100µM (**J-L**) and high toxicity 120µM (**M-O**) (at 10x magnification)

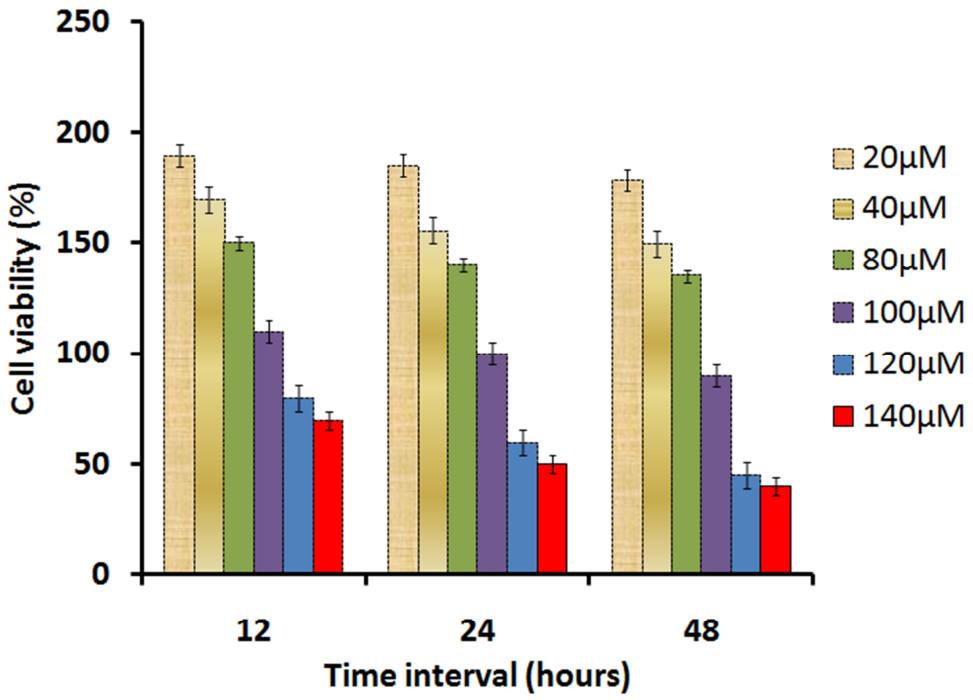
5.2.4 LD_{50} concentration of $CoCl_2$ is 120μM

The 120μM concentration of $CoCl_2$ exhibited heavy toxicity on SH-SY5Y cells which was clearly evident after 12 hours of administration in the form of almost complete distortion of neuronal cell morphology. Further, cell morphology was lost after 24 hours and more than 80% cells were dead post 48 hours of 120μM dose administration (**Figure 5.2**). Thus, after repeating the experiment in triplicates thrice, 120μM concentration of $CoCl_2$ was taken as the LD_{50} value in SH-SY5Y cells. Furthermore, the $CoCl_2$ toxicity was extremely high at 140μM concentration and immediate deformation of neuronal cells with cell death was visible. Moreover, the morphology and viability was completely lost at 12 hours and cells were floating after 24 hours (**Figure 5.2**).

5.2.5 Effect of $CoCl_2$ dose on cell viability

To determine the effect of $CoCl_2$ on cell viability, the trypan blue exclusion test was performed in all the above mentioned sets of experiments. Accordingly, the number of live (unstained) and dead (stained) cells were counted in a hemocytometer and percent cell viability determined in triplicate set of experiments. The results in the form of statistically calculated values were plotted on a graph. The cell viability was found to decrease with increasing concentration of $CoCl_2$, further validating the dose-dependent effect of $CoCl_2$ toxicity on SH-SY5Y cells (**Graph 5.1**).

Next, we checked the neuroprotective potential of biomolecules (*Naringenin* and *Quercetin*) which have been described in both the *Chapter III* and *Chapter IV* as these molecules regulate the impaired expression of angiogenic markers; in alleviating $CoCl_2$ induced toxicity and to check its efficacy on cell viability and on the expression of various angiogenic markers. The results are summarized in the next section.



Graph 5.1: Dose-dependent effect of $CoCl_2$ toxicity on SH-SY5Y cells. As the dose of $CoCl_2$ was increased, cell viability attenuated significantly as concluded from trypan blue exclusion test (n=3)

5.3 BIOMOLECULES REVERSE $CoCl_2$-INDUCED TOXICITY IN SH-SY5Y CELLS

5.3.1 Dose-dependent effect of *Naringenin* on $CoCl_2$-induced toxicity in SH-SY5Y cells

The concentration gradient of *Naringenin* was prepared in increasing order of 25µM, 50µM, 75µM and100µM respectively. Next, the LD_{50} value of $CoCl_2$ which was determined as 120µM in preceding experiment was administered to SH-SY5Y cells for 24 hours. The cell death incurred upon $CoCl_2$ administration at 120µM was determined through trypan blue cell viability assay and observed morphological deformations. Next, the varying concentrations of *Naringenin* were used to study its protective effect on $CoCl_2$ induced toxicity on SH-SY5Y cells in period of 24 hours. The results obtained are summarized below.

5.3.2 *Naringenin* protects against $CoCl_2$-induced toxicity at 50µM concentration

Naringenin restored almost 50% cells which were non-viable post $CoCl_2$ induced toxicity in SH-SY5Y cells. The rounded morphology of $CoCl_2$-treated cells was transformed into well defined neurite-like structures upon 50µM *Naringenin* treatment **(Figure 5.3)**. Further, the cell viability was greatly enhanced and these results are corroborated with cell viability assays. Therefore, the present study observed well marked protective effect of 50µM *Naringenin* against $CoCl_2$ induced toxicity in SH-SY5Y cells.

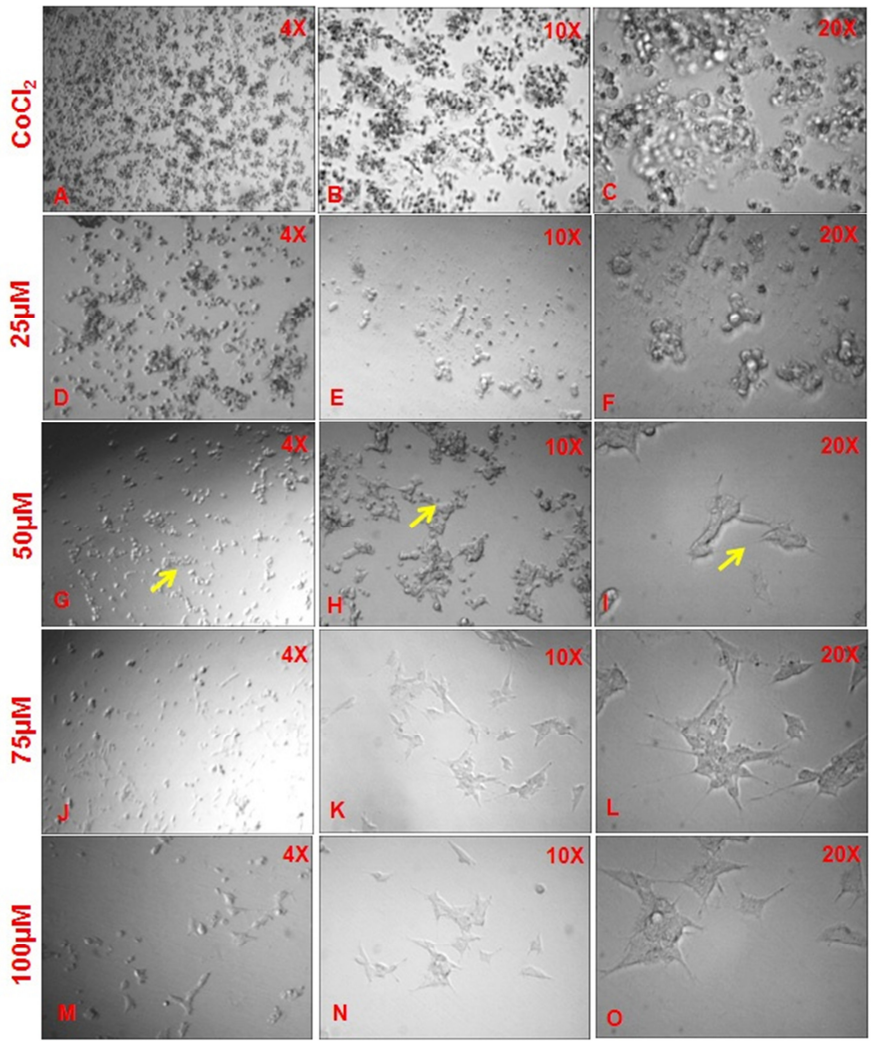


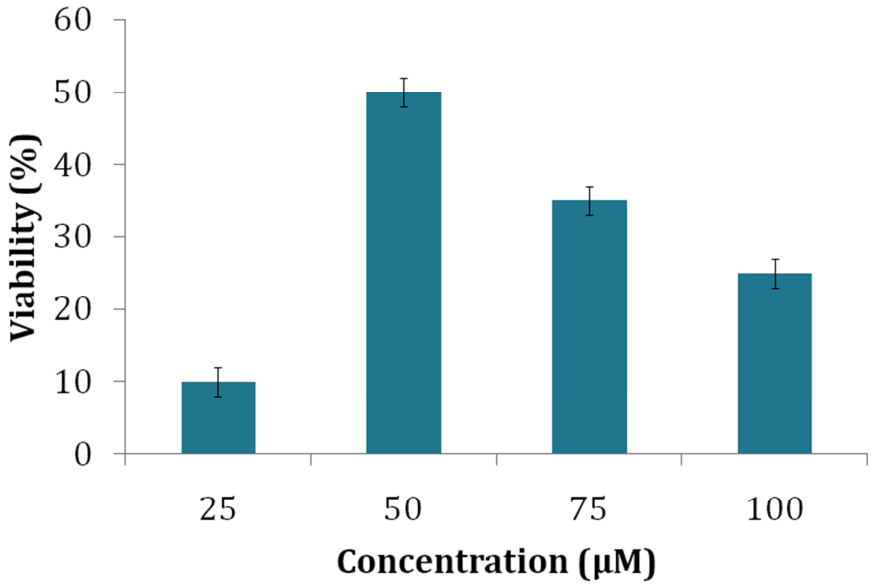
Figure 5.3: *Naringenin* is protective at 50 µM and revived more than 50% cells at this dose. Moreover, *Naringenin* reverses $CoCl_2$ toxicity at 75µM concentration (**J-L Lower panel**). At this dose all the cells were rescued and cell viability was greatly enhanced (4x-20x magnification)

5.3.3 *Naringenin* reverses $CoCl_2$-induced toxicity at 75µM concentration

The concentration of *Naringenin* at which there was almost complete reversal of $CoCl_2$-induced toxicity was found to be 75µM. The neuronal morphology was very well defined and long neurites could be seen after treatment with this dose (**Figure 5.3**). Thus, *Naringenin* rescued neuronal cells from $CoCl_2$ toxicity at 75µM.

5.3.4 Effect of dose of *Naringenin* on cell viability

The protective effect of *Naringenin* on cell viability in $CoCl_2$ induced toxicity was determined through trypan blue exclusion test. Post 24 hours of $CoCl_2$ administration, cells were treated with *Naringenin* for 24 hours. Next, the trypan blue test was performed for each experimental and control group. Accordingly, the number of live (unstained) and dead (stained) cells were counted in a hemocytometer and percent cell viability determined in triplicate set of experiments. The results in the form of statistically calculated values were plotted on a graph **(Graph 5.2)**. To summarise, *Naringenin* treatment increased cell viability in a dose-dependent manner in $CoCl_2$ induced toxicity in SH-SY5Y cells.



Graph 5.2: *Naringenin* increased cell viability in a dose-dependent manner against $CoCl_2$ toxicity. At 50µM *Naringenin* reversed cell toxicity and greatly enhanced cell viability (n=3)

Further, we wanted to check the effect of *Quercetin* on $CoCl_2$ induced toxicity in SH-SY5Y cells. For this purpose we performed same set of experiments and the results obtained are summarized in the succeeding section.

5.3.5 Dose-dependent effect of *Quercetin* in $CoCl_2$-induced toxicity

The concentration gradient of *Quercetin* was prepared in increasing order of 25μM, 50μM, 75 μM and 100μM respectively. Next, 120μM $CoCl_2$ was administered to SH-SY5Y cells for 24 hours. The cell death incurred upon $CoCl_2$ administration was determined through trypan blue cell viability assay. Next, the effect of varying concentrations of Quercetin on $CoCl_2$ induced toxicity was studied and obtained results are summarized below.

5.3.6 *Quercetin* does not attenuate $CoCl_2$ induced toxicity at 25μM concentration

$CoCl_2$ treated cells were incubated with 25μM *Quercetin* for 12-48 hours. The effect of *Quercetin* was observed after 12 hours, 24 hours and 48 hours. However, *Quercetin* had no effect on $CoCl_2$ induced toxicity in SH-SY5Y cells at 25μM concentration **(Figure 5.4)**. The morphology of cells as well as the cell viability assessed through trypan blue test and remained unchanged on treatment with 25μM *Quercetin* (**Graph 5.3**).

5.3.7 *Quercetin* shows slight activity at 50μM concentration

The dose of *Quercetin* was further increased to study its effect in $CoCl_2$ induced toxicity. Accordingly, 50μM *Quercetin* was used to treat $CoCl_2$ administered cells. After 24 hours it was observed that these doses of *Quercetin* had a very slight effect on cells. Moreover, a very few viable cells were observed in the total population of $CoCl_2$ induced dead cells (**Figure 5.4**). Further, the cell viability was very slightly increased at 50μM *Quercetin* concentration. Interestingly, on further increasing the dose of *Quercetin* to 75μM, improved cell viability as compared to the preceding dose was observed (**Figure 5.4**).

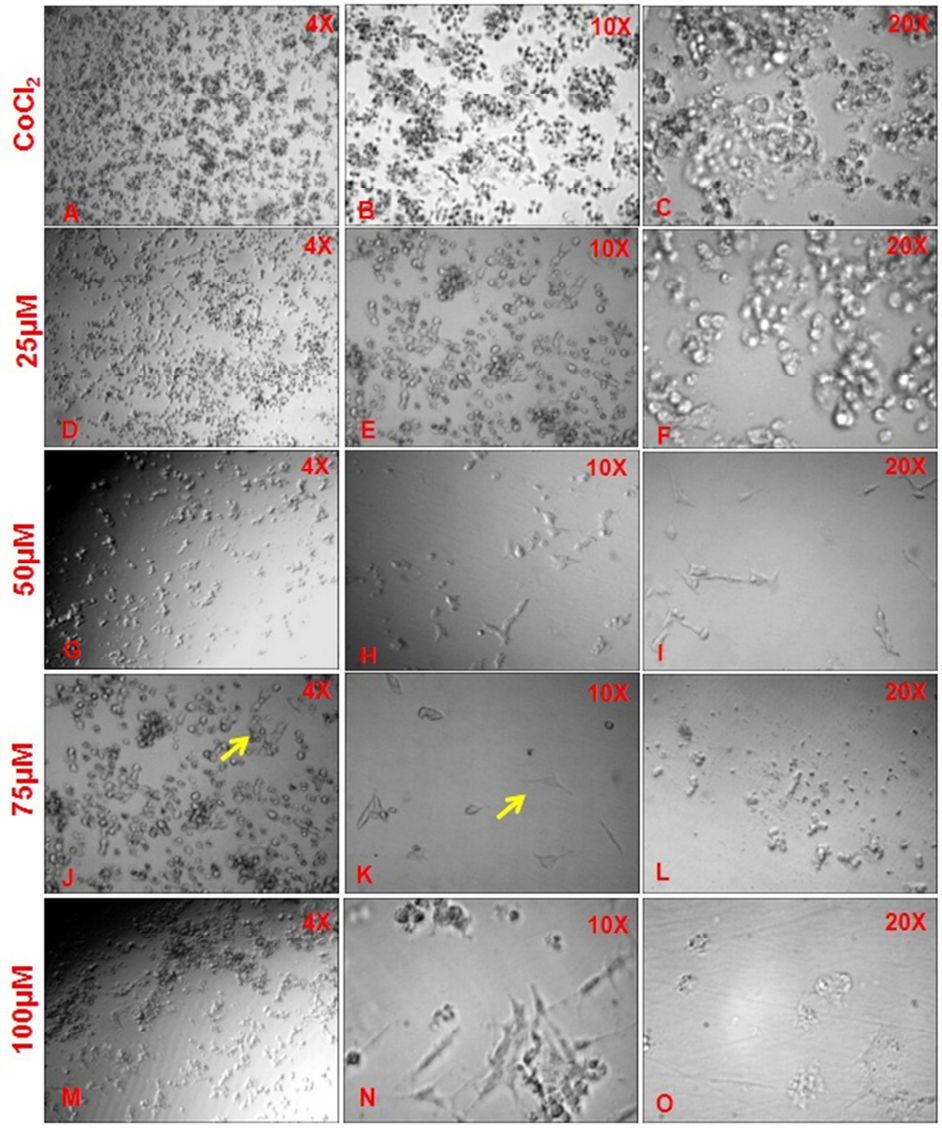
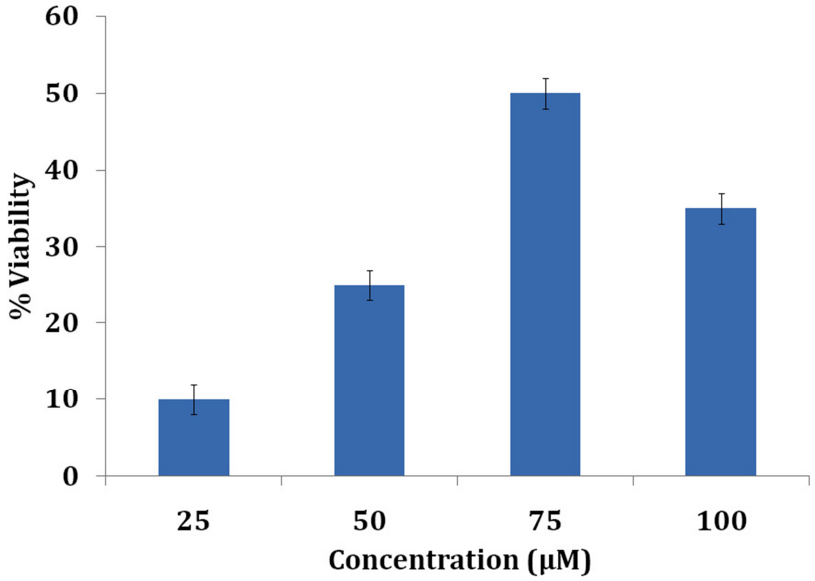


Figure 5.4: *Quercetin* had no effect on $CoCl_2$ toxicity at 25µM concentration. However, *Quercetin* showed very slight activity at 50µM concentration. Although *Quercetin* showed significant result at 75µM concentration in the total population of dead cells

5.3.8 Effect of dose of *Quercetin* on cell viability

The protective effect of *Quercetin* on cell viability in $CoCl_2$ induced toxicity was determined through trypan blue exclusion test. $CoCl_2$ treated cells were incubated with varying concentration of *Quercetin*. Next, the trypan blue test was performed for each experimental and control group. Further, the number of live (unstained) and dead (stained) cells were counted in a hemocytometer and percent cell viability determined in

triplicate set of experiments. The results in the form of statistically calculated values were plotted on a graph (**Graph 5.3**). To summarize, *Quercetin* treatment also exhibited significant increase in cell viability in $CoCl_2$ induced toxicity in SH-SY5Y cells.



Graph 5.3: *Quercetin* increased cell viability in a dose-dependent manner against $CoCl_2$ toxicity. At 75µM *Quercetin* reversed cell toxicity. Cell viability as a measure of *Quercetin* doses showing significant results (n=3)

5.4 PROTEIN PROFILING STUDY FOR BOTH ANGIOGENIC AND PROLIFERATION MARKERS

5.4.1 $CoCl_2$ induced upregulation of HIF-1α and VEGF in SH-SY5Y cell line

HIF-1α and VEGF are angiogenic and proliferation markers which normally remain suppressed in normoxic condition. Upon hypoxic induction, these markers get activated and trigger downstream signaling associated with deprived O_2 and lead to the cellular dysfunction. Accordingly, the expression level of both HIF-1α and VEGF was found to be similar as compared to control cells (Normoxia). However, upon $CoCl_2$ treatment increased level of HIF-1α and VEGF as indicative of hypoxic induction was observed. $CoCl_2$ led increase in these markers may be contributed to accumulation of Aβ and tau phosphoryaltion, which ultimately lead to hamper neuronal network and cause neuronal dysfunction.

5.4.2 *Naringenin* reduced the level of HIF-1α and VEGF

Naringenin treatment attenuated the levels of HIF-1α and VEGF which were upregulated in response to $CoCl_2$ exposure (**Figure 5.5**). The decrease in levels of these markers may be mediating significant acceleration in cell viability upon *Naringenin* treatment in SH-SY5Y cells. Further, this decrease may signify curtail in hypoxia induced neuronal death.

5.4.3 *Quercetin* reduced the level of HIF-1α and VEGF

Further similar result was found in case of *Quercetin* treatment. Administration of *Quercetin* attenuated the levels of both HIF-1α and VEGF which were upregulated in response to $CoCl_2$ exposure (**Figure 5.5**). The decrease in levels of these markers may be mediating significant acceleration in cell viability upon *Quercetin* treatment in SH-SY5Y cells. Further, the obtained result may signify curtail in hypoxia induced cellular damage.

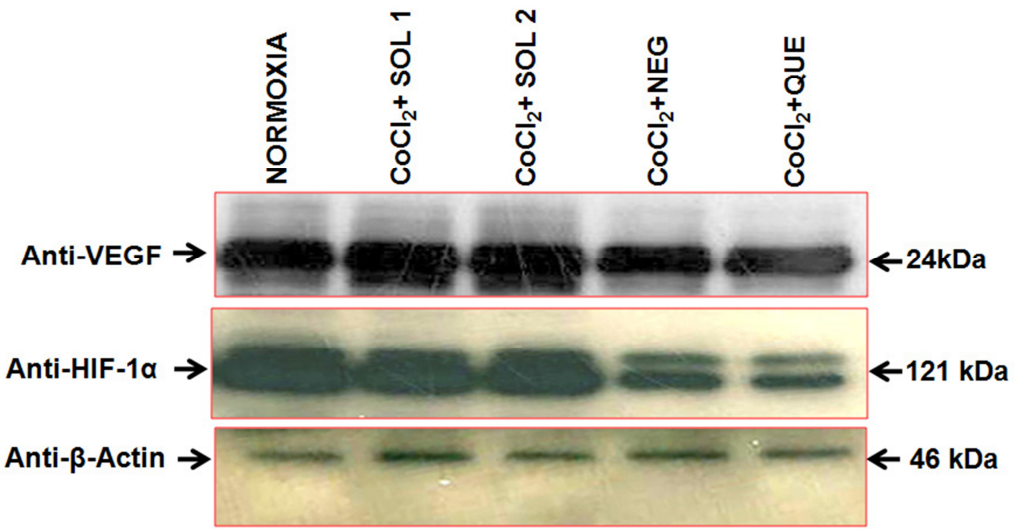


Figure 5.5: Protein expression of HIF-1α and VEGF subsequent to $CoCl_2$ administration and on biomolecules treatment in SH-SY5Y cell line

After noticing profound expression of these angiogenic and proliferation markers in SH-SY5Y cells, we wanted to check the expression of these markers in HEK-293 cell line. Since, the increased plasma VEGF expressions have been observed in chronic kidney disease (CKD) patients.

5.4.4 $CoCl_2$ induced upregulation of HIF-1α and VEGF in HEK-293 cell line

The expression level of both HIF-1α and VEGF was found to be similar as compared to control cells (Normoxia). However, upon $CoCl_2$ treatment increased the level of HIF-1α and VEGF as indicative of hypoxic induction was observed in HEK-293 cell line as well. $CoCl_2$ led increase in these markers may be contributed to increase in VEGF expressions which may cause CKD associated with atherosclerosis and sepsis.

5.4.5 *Naringenin* reduced the level of HIF-1α and VEGF

Naringenin treatment attenuated the levels of HIF-1α and VEGF in HEK-293 cell line which were upregulated in response to $CoCl_2$ exposure (**Figure 5.6)**. The decrease in levels of these markers may be directing remarkable acceleration in cell viability upon *Naringenin* treatment in HEK-293 cell line. Further, this decrease may play a decisive role in curtailing hypoxia induced cellular damage.

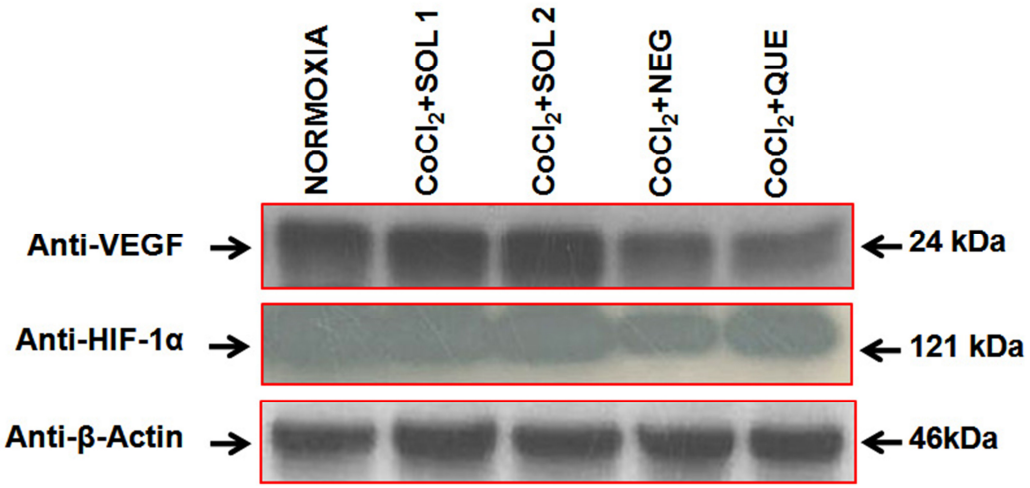


Figure 5.6: Protein expression of HIF-1α and VEGF subsequent to $CoCl_2$ administration and on biomolecules treatment in HEK-293 cell line

5.4.6 *Quercetin* reduced the level of HIF-1α and VEGF

Further, *Quercetin* treatment also attenuated the levels of both HIF-1α and VEGF in HEK-293 cell line (**Figure 5.6)**. The decrease in levels of these markers may be

mediating notable acceleration in cell viability upon *Quercetin* treatment in HEK-293 cell line. Finally, this result highlights possible therapeutic potential of *Quercetin* in alleviating hypoxia induced cellular death.

5.5 DISCUSSION

The previous chapters discussed about the potential role of HIF-1α and VEGF regulating compounds in ameliorating hypoxia induced cellular damage. Out of the three compounds shortlisted in *chapter III* and *chapter IV*, the protective potential of *Naringenin* and *Quercetin* in alleviating hypoxia mediated cell death was evaluated in the present chapter. The $CoCl_2$ which is widely used in mimicking hypoxic condition was used to induce toxicity in both SH-SY5Y and HEK-293 cell line. The toxicity assay shows dose-dependent and time-dependent effect of $CoCl_2$ in inducing neuronal cell death in SH-SY5Y cells and cell viability significantly decreased with increasing dose of $CoCl_2$. Moreover, the LD_{50} value of $CoCl_2$ was determined at 120μM concentration in present study. Further, treatment with *Naringenin* rescued cells against $CoCl_2$ induced toxicity at 50μM dose and exhibited dose-dependent and time-dependent protection in increasing cell viability. The protein expression level of angiogenic and proliferation markers subsequent to $CoCl_2$ administration and on *Naringenin* treatment was checked through western blotting. The results show that *Naringenin* attenuated the level of both HIF-1α and VEGF (indicative of hypoxic induction) which were upregulated on $CoCl_2$ treatment in both SH-SY5Y and HEK-293 cell line. Likewise, *Quercetin* administration rescued cells against $CoCl_2$ induced toxicity at 75μM dose in SH-SY5Y cells and exhibited dose-dependent and time-dependent protection in increasing cell viability. Further, the protein expression level of HIF-1α and VEGF to $CoCl_2$ administration and on *Quercetin* treatment in both the SH-SY5Y and HEK-293 cell line was checked through western blotting. The results demonstrate that *Quercetin* attenuated the level of both HIF-1α and VEGF which were upregulated on $CoCl_2$ treatment. Therefore, it can be advocated from the given results that *Naringenin* and *Quercetin* may be rescuing neuronal cells and kidney cells against $CoCl_2$ induced toxicity through the inhibition of both HIF-1α and VEGF. The detailed mechanism of such promising interaction can be validated through further studies.

Chapter VI

Summary, Discussion and Future Perspective

CHAPTER VI

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVE

Oxygen (O_2) is pivotal for cell survival. Hence, tissue oxygenation is quite important for all physiological functions including but not limited to, cell growth, respiration and metabolism. Although, medical complications such as asthma, cardiac arrest, pneumonia and stroke cause the body to be deprived of adequate O_2 or a condition called tissue hypoxia. Nowadays, pollution has also been a causative factor for making tissue to be deprived of adequate O_2. Moreover, extensive athletic training, hiking at high altitudes and diving underwater are also responsible for hypoxic condition in the body. In turn, hypoxia can be defined as the insufficiency in the bioavailability of O_2 to the tissues of the body. Further, exposure to high-altitude results in hypobaric hypoxia which is considered as an acute physiological stress. This condition frequently leads to high-altitude illnesses such as HACE, HAPE and hypoxic muscle weakness [Sarkar *et al.,* 2012]. Similarly, chronic exposure to hypoxia has also been reportedly involved in defective vessels formation. Such vascular abnormalities lead to altered blood flow, reduced nutrient delivery and entry of otherwise restricted infiltrates thereby limiting oxygen availability to the brain and cause neurological disabilities.

Amongst many factors amenable for the cellular damage under hypoxia, altered expression of HIF-1α and VEGF have been recently identified as the cause of cellular death. The elevated expression of VEGF in AD under hypoxia causes abnormal vessel branching, increased vessel diameter, irregular basement membrane and an increased endothelial cell proliferation. Such vascular abnormalities may lead to altered blood flow, reduced nutrient delivery and entry of otherwise restricted infiltrates [Iyer *et al.,* 1998]. Moreover, angiogenesis triggers the expression of various pro-angiogenic markers, including TGF, VEGF and TNF [Greer *et al.,* 2012]. The angiogenic activation of all these factors under hypoxic insult leads to agglomeration of Aβ peptides and secretion of a neurotoxic peptide that usually hampers cortical neuronal

activity and results in the pathophysiology of AD [Semenza 2002]. The activity of VEGF protein is triggered under hypoxia through HIF-1α and found to be upraised in the frontal and para-hippocampal cortex in AD brain [Zhang *et al.,* 2011]. While in PD, mutations in DJ-1 promoted VHL mediated degradation of the α-subunit (HIF-1α) which further leads to MPP+-induced toxicity in neurons [Ke and Costa 2006]. The normal response to hypoxia involves two crucial pathways, in particular: the HIF-1α pathway (which notably controls the synthesis of VEGF and the nuclear factor NF-κB pathway (responsible for the production of inflammatory mediators, including prostaglandin E2 (PGE2)). Any alterations in these pathways contribute to the fatal complication of ALS. Defects in VEGF gene expression and deregulation of the HIF-1α pathway are selectively and markedly altered in ALS patients. In case of diabetic nephropathy patients, the VEGF levels were found to be increased and blocking VEGF or VEGF receptor ameliorated diabetic nephropathy in animal models. Increased plasma VEGF expressions have also been observed in CKD patients. Additionally, lethal effects of VEGF have been reported in atherosclerosis and sepsis, which are common hurdle in CKD patients (Doi *et al.,* 2010). Given the outcome of substantial cellular damage caused by deprived O_2, it is crucial to design therapeutics targeting hypoxic signaling through controlling the expression of both HIF-1α and VEGF. Such a strategy appears promising and is more reasonable in the current study given that both HIF-1α and VEGF is the central molecule involved in cellular damage under hypoxia.

In the present work, we first aimed to explore for both the HIF-1α and VEGF modulating compounds in cellular damage under hypoxia. For this, we carried out extensive literature survey with the keywords 'HIF-1α and VEGF associated regulatory biomolecules' and 'Hypoxia'. Further we prepared a list of thirteen compounds on the basis of extensive literature survey. Next, we employed various *in silico* tools and techniques for screening of these compounds on drug-likeliness parameters. The compounds were assessed using three parameters namely; Lipinski filter, Ghose and Veber rules and those which passed these filters were then analyzed for pharmacokinetic properties. Based on various pharmacokinetic parameters such as aqueous solubility, lipophilicity, GI permeability,

bioavailability and BBB permeability scores, the final candidates amongst thirteen compounds were obtained. These compounds which passed all the filters and showed good pharmacokinetics included *Naringenin, Sesamol* and *Quercetin*. Next, we performed structural analysis of target proteins HIF-1α and VEGF for adding therapeutics potential of these screened compounds.

Naringenin, Sesamol and *Quercetin* depicted crucial role in regulating both the HIF-1α and VEGF levels based on interaction energies of molecular docking study. These results led to speculations that these compounds may have strong potential in inhibiting downstream signaling associated with both HIF-1α and VEGF as impaired expression of these proteins lead to cellular damage under deprived O_2. Further, the protective action of these compounds in ameliorating hypoxia induced cellular damage may be mediated through the HIF-1α and VEGF inhibition.

Finally, we tested the protective potential of two of these shortlisted biomolecules; *Naringenin* and *Quercetin* in SH-SY5Y neuroblastoma cell line. The neurotoxin $CoCl_2$ which is widely used to mimic hypoxic conditions was used to induce toxicity in the given cell line. Firstly, we tested the dose-dependent and time-dependent effect of $CoCl_2$ toxicity on cell viability and determined the LD_{50} value of $CoCl_2$ which was found to be 120μM in a 24 hour time period in the given study. Moreover, we also found that $CoCl_2$ exerts toxicity in a dose dependent manner and cell viability decreases significantly and markedly with increasing dose. Next, we studied the dose-dependent and time-dependent efficacy of *Naringenin* against $CoCl_2$ induced toxicity in SH-SY5Y cell line. Accordingly, our study outlined protective potential of *Naringenin* at a dose of 50μM where reversal of $CoCl_2$ induced toxicity in SH-SY5SY cells was observed. Similarly we studied the dose-dependent and time-dependent efficacy of *Quercetin* against $CoCl_2$ induced toxicity in SH-SY5Y cell line. Accordingly, our study outlined protective potential of *Quercetin* at a dose of 75μM and at this particular dose reversal of $CoCl_2$ induced toxicity in neuroblastoma cell line was noticed. Further, both *Naringenin* and *Quercetin* exhibited dose dependent efficacy to a greater extent and cell viability was significantly enhanced with increasing dose of these biomolecules.

Further, the level of these angiogenic/pro-angiogenic and cell proliferation markers was checked subsequent to $CoCl_2$ administration and on treatment with biomolecules in SH-SY5Y and HEK-293 cells. Our results showed that both *Naringenin* and *Quercetin* reduced the level of HIF-1α and VEGF to some extent thus showing protective role in cellular damage under hypoxia. To summarize, both *Naringenin* and *Quercetin* attenuated hypoxia induced cellular damage by reducing the level of angiogenic/pro-angiogenic and cell proliferation markers. These observations have opened up a new avenue of therapeutics for further exploration through *in vivo* hypoxia models in cellular damage.

The present study can be extended to check the expression of different heat shock proteins (HSPs) and ubiquitin in cellular damage under hypoxia. Additionally, the role apoptotic pathways and their downstream signaling molecules in O_2 deprived cells can be further explored to validate the mode of action of screened biomolecules in promoting cell-survival in hypoxia induced cellular death.

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Appendices

Appendix I

REAGENTS AND BUFFERS

(1) 1X Phosphate-buffered saline (PBS; pH-7.4)

NaCl : 8g

KCl : 0.2g

Na_2HpO_4 : 1.44g

KH_2PO_4 : 0.24g

ddH_2O : Adjust the volume to one litre and stored at room temperature

(2) 1X Phosphate-buffered saline (PBS; pH-7.2)

NaCl : 8g

KCl : 0.2g

Na_2HpO_4 : 1.44g

KH_2PO_4 : 0.2g

ddH_2O : Adjust the volume to one litre and stored at room temperature

(3) Laemmli sample buffer (SB; pH-6.8)

SDS : 1.6g

Glycerol : 8mL

β-ME : 4mL

Bromophenol blue : 0.0016g

ddH_2O : Adjust the volume to 40mL and stored at 4ºC/-20ºC

(4) Acrylamide mix

Acrylamide : 29g

Bis Acrylamide : 1g

ddH_2O : Adjust the volume to 100mL and stored at 4ºC

(5) 10% Ammonium persulfate (APS)

APS : 0.1g

ddH_2O : Adjust the volume to 1mL and stored at -20ºC

(6) 1 M Tris Cl (pH- 6.8)

Trizma : 12.114g

ddH_2O : Adjust the volume to 100mL and stored at 4ºC

(7) 1.5 M Tris Cl (pH- 8.8)

Trizma : 18.17g

ddH_2O : Adjust the volume to 100mL and stored at 4ºC

(8) 10% SDS

SDS : 10g

ddH_2O : Adjust the volume to 100mL and stored at room temperature

(9) Tris Glycine (pH-8.3)

Trizma : 3.02g

Glycine : 18.768g

SDS : 1g

ddH_2O : Adjust the volume to one litre and stored at room temperature

(10) 10% Resolving gel (pH-8.8)

ddH_2O : 1.9mL

30% Acrylamide mix : 1.8mL

1.5 M Tris (pH-8.8) : 1.3mL

10% SDS : 0.05mL

10% APS : 0.05mL

TEMED : 0.004mL

(11) 5% Stacking gel (pH-6.8)

ddH_2O : 2.1mL

30% Acrylamide mix : 0.5mL

1 M Tris (pH-6.8) : 0.38mL

10% SDS : 0.03mL

10% APS : 0.035mL

TEMED : 0.004mL

(12) Transfer buffer (TB; pH-8.3)

Trizma : 2.907g

Glycine : 14.413g

Methanol : 200mL

ddH_2O : Adjust the volume to one litre and stored at 4ºC

(13) 5% blocking buffer

Skimmed milk : 5g

TBST : Adjust the volume to 100mL and stored at 4ºC

(14) Tris-buffered saline tween 20 (TBST; pH-7.4)

NaCl : 8.8g

KCl : 0.2g

Trizma : 3g

Tween 20 : 2mL

ddH_2O : Adjust the volume to one litre and stored at room temperature

(15) Stripping buffer (pH-6.8)

Tris Cl : 0.7571g

10% SDS : 20mL

β-ME : 700µL

ddH_2O : Adjust the volume to 100mL and stored at room temperature

(16) Ponceau S Staining Solution (0.1%(w/v) Ponceau S in 5%(v/v) acetic acid)

Ponceau S : 0.1g

Acetic acid : 50ml

Make up to 100mL with ddH2O and stored at 4°C

Appendix II

LIST OF CHEMICALS AND ANTIBODIES

List of Chemicals

S.No.	Common Name	Chemical Formula	Product number	Company
1	Acrylamide	CH_2=CHCONH$_2$	A3553	Sigma-Aldrich
2	Acetic acid glacial	CH_3COOH	320099	Sigma-Aldrich
3	Ammonium persulfate (APS)	$(NH4)_2S_2O_8$	A7460	Sigma-Aldrich
4	Quercetin	$C_{15}H_{10}O_7$ $2H_2O$	A3145	Sigma-Aldrich
5	Bis Acrylamide	$(H_2C$=CHCONH$)_2CH_2$	146072	Sigma-Aldrich
6	Bovine serum albumin (BSA)	-	A7906	Sigma-Aldrich
7	Bromophenol blue	$C_{19}H_{10}Br_4O_5S$	32712	Sigma-Aldrich
8	Dimethyl sulfoxide (DMSO)	$(CH_3)_2SO$	D8418	Sigma-Aldrich
9	Dulbecco's Modified Eagle Medium (DMEM)	-	11965092	Invitrogen
10	Ethanol	CH_3CH_2OH	459844	Sigma-Aldrich
11	Ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid	$[CH_2OCH_2CH_2N(CH_2CO_2H)_2]_2$	E3889	Sigma-Aldrich
12	Ethylene Diamine tetraacetic acid disodium salt dehydrate (EDTA)	$C_{10}H_{14}N_2Na_2O_8$ $2H_2O$	E5134	Sigma-Aldrich
13	Formaldehyde solution	HCHO	F8775	Sigma-Aldrich
14	GBX developer/replenisher	-	P7042	Sigma-Aldrich
15	GBX fixer/replenisher	-	P7167	Sigma-Aldrich
16	Glycerol	$C_3H_8O_3$	G5516	Sigma-Aldrich
17	Glycine	NH_2CH_2COOH	G8898	Sigma-Aldrich
18	Coomassie Brilliant Blue (CBB)	$C_{45}H_{44}N_3NaO_7S_2$	27815	Sigma-Aldrich
19	Hydrochloric acid	HCl		Sigma-Aldrich
20	Lithium carbonate	Li_2CO_3	62470	Sigma-Aldrich
21	Methanol	CH_3OH	34860	Sigma-Aldrich
22	*N,N,N′,N′*-Tetramethyl ethylenediamine (TEMED)	$(CH_3)_2NCH_2CH_2N(CH_3)_2$	T7024	Sigma-Aldrich

S.No.	Common Name	Chemical Formula	Product number	Company
23	Naringenin	$C_{15}H_{12}O_5$	N5893	Sigma-Aldrich
24	Penicillin streptomycin	-	P4333	Sigma-Aldrich
25	Phenylmethylsulfonyl fluoride (PMSF)	$C_7H_7FO_2S$	P7626	Sigma-Aldrich
26	Ponceau S	$C_{22}H_{12}N_4Na_4O_{13}S_4$	P3504	Sigma-Aldrich
27	Potassium chloride	KCl	P9541	Sigma-Aldrich
28	Potassium phosphate monobasic	KH_2PO_4	P5655	Sigma-Aldrich
29	Protease inhibitor	-	4693116	Roche
30	Skimmed milk	-	70166	Sigma-Aldrich
31	Sodium chloride	NaCl	S3014	Sigma-Aldrich
32	Sodium dodecyl sulfate (SDS)	$CH_3(CH_2)_{11}OSO_3Na$	L3771	Sigma-Aldrich
33	Sodium orthovanadate	Na_3VO_4	S6508	Sigma-Aldrich
34	Sodium phosphate dibasic	Na_2HPO_4	S3264	Sigma-Aldrich
35	Sodium phosphate monobasic monohydrate	NaH_2PO_4 H_2O	S9638	Sigma-Aldrich
36	Sodium pyrophosphate tetrabasic decahydrate	$Na_4P_2O_7$ $10H_2O$	221368	Sigma-Aldrich
37	Triton-X 100	-	T8787	Sigma-Aldrich
38	Trizma	$NH_2C(CH_2OH)_3$	T6066	Sigma-Aldrich
39	Tween-20	-	274348	Sigma-Aldrich
40	β-Mercaptoethanol (β-ME)	$HSCH_2CH_2OH$	M3148	Sigma-Aldrich

List of Antibodies

S.No.	Name of Antibodies	Product Number	Company
1	β-Actin	SC-47778	Santacruz
2	HIF-1α	SC-10790	Santacruz
3	VEGF	SC-7269	Santacruz
4	Fluorescent Secondary Antibodies (Anti- rabbit IGg)	NL007	R&D System
5	Fluorescent Secondary Antibodies (Anti- mouse IGg)	NL004	R&D System

Appendix III

LIST OF INSTRUMENTS

CO_2 incubator	:	Thermo Fisher Scientific
Chest freezer	:	Blue Star CHF150B
Autoradiography Hypercassette	:	Amersham Biosciences RPN11642
Inverted laboratory microscope	:	Magnus
Gel Rocking Shaker	:	Tarsons 4080. Rockymax
Minispin Microcentrifuge	:	Eppendorf Centrifuge Z606235
Refrigerator	:	Blue Star
SDS PAGE and Western Blot unit	:	BioRad Mini-PROTEAN Tetra Cell, Mini Trans-Blot Module, and PowerPac Basic Power Supply #165-8033
Water bath	:	JULABO
Biological Safety Cabinet	:	Esco Labculture® Class II Type A2

Publications

PUBLICATIONS

Publications from Thesis

[Cumulative impact factor: 34.88; cumulative citations: 84; h-index: 5]

1. **Niraj Kumar Jha** and Pravir Kumar **(2017***) "Molecular docking studies for the comparative analysis of different biomolecules to target Hypoxia inducible factor-1α".* ***International Journal of Applied Pharmaceutics***. Vol 9 Issue 4, July 2017. **[Impact factor: 0.88]** [https://innovareacademics.in/journals/index.php/ijap/article/view/19505]

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3. **Niraj Kumar Jha**, Saurabh Kumar Jha, Dhiraj Kumar, Noopur Kejariwal, Renu Sharma, Rashmi K Ambasta and Pravir Kumar (**2015**), *Impact of IDE and Neprilysin in Alzheimer's disease biology: Characterization of putative cognates for therapeutic applications*. J Alzheimer's Dis. 2015;48(4):891-917. [**Impact factor: 3.72]**. [http://www.ncbi.nlm.nih.gov/pubmed/26444774]

4. **Niraj Kumar Jha**, Saurabh Kumar Jha, Rohan Kar, Rashmi K Ambasta and Pravir Kumar (2014), *Role of oxidative stress, ER stress and Ubiquitin Proteasome system in neurodegeneration,* ***MOJ Cell Science and Report****.01(2). [http://m*edcraveonline.com/MOJCSR/MOJCSR-01-00010.pdf]

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2. Saurabh Kumar Jha, **Niraj Kumar Jha**, Pravir Kumar and Rashmi K Ambasta **(2016)**, *Molecular Chaperones and Ubiquitin Proteasome System in Tumor Biogenesis: An Overview,* ***Journal of Cell Biology and Cell Metabolism***, 3: 010 [http://heraldopenaccess.us/fulltext/Cell-Biology-&-Cell-Metabolism/Molecular-Chaperones-and-Ubiquitin-Proteasome-System-in-Tumor-Biogenesis-An-Overview.php]

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4. Saurabh Kumar Jha, **Niraj Kumar Jha**, Dhiraj Kumar, Renu Sharma, Abhishek Shrivastava, Rashmi K Ambasta and Pravir Kumar (**2016**), *Stress induced synaptic dysfunction and neurotransmitter release in Alzheimer's disease: Can neurotransmitter and Neuromodulator be a potential therapeutic target?*, ***Journal of Alzheimer's Disease***, 2016; 57(4):1017-1039, [**Impact factor: 3.72]** [https://www.ncbi.nlm.nih.gov/pubmed/27662312]

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6. Rashmi K. Ambasta, Saurabh Kumar Jha, Dhiraj Kumar, Renu Sharma, **Niraj Kumar Jha**, and Pravir Kumar (**2015**) *Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules,* ***Journal of Translational Medicine*****, 13:307, [Impact factor: 3.96]** [http://www.ncbi.nlm.nih.gov/pubmed/26385094]

7. [http://www.ncbi.nlm.nih.gov/pubmed/26385094] Pravir Kumar, **Niraj Kumar Jha**, Saurabh Kumar Jha, Karunya Ramani and Rashmi K Ambasta (**2015**), *Tau ph**o**sphorylation, molecular chaperones, Ubiquitin E3 ligase: clinical relevance in Alzheimer's disease*, ***Journal of Alzheimer's Disease, 2015;43(2):341-61; [Impac*t factor: 3.72]** [http://www.ncbi.nlm.nih.gov/pubmed/25096626]

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2. Alka Raina, Saurabh Kumar Jha, **Niraj Kumar Jha**, Dhiraj kumar, Rashmi K Ambasta and Pravir Kumar (**2015**), *Putative transcription factor binding elements of ubiquitin E3 ligase in neurodegenerative disorders,* 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), December 19-21, 2015, NEHU Shillong [Poster presentation]

3. Abhisekh Srivastava, Puspendramani Mishra, Dhiraj kumar, Saurabh Kumar Jha, **Niraj Kumar Jha**, Rashmi K Ambasta and Pravir Kumar (**2015**), *Relevance of terpenoids and alkaloids in neuroprotection,* 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), December 19-21, 2015, NEHU Shillong [Poster presentation]

4. Swati Sharan, **Niraj Kumar Jha**, Saurabh Kumar Jha, Dhiraj kumar, Rashmi K Ambasta and Pravir Kumar (**2015**), *Post-translational modification mechanism in Parkinson's disease pathology,* 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), December 19-21, 2015, NEHU Shillong [Poster presentation]

5. Minal Singh, **Niraj Kumar Jha**, Saurabh Kumar Jha, Dhiraj kumar, Rashmi K Ambasta and Pravir Kumar (**2015**), *In silico characterization of holo AβPP promoter and its transactivation modules* 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), December 19-21, 2015, NEHU Shillong [Poster presentation]

6. Abhishek Srivastava, Puspendra M Mishra, Saurabh Kumar Jha, **Niraj Kumar Jha**, Rashmi K Ambasta and Pravir Kumar (2015), *In silico analysis cannaboids in neurodegeneration,* 6th World congress on biotechnology, New Delhi, October 5th-7th, *J Biotechnol Biomater* 2015, 5:6

7. Pushpendra Mani Mishra, Abhishek Srivastava, **Niraj Kumar Jha**, Rashmi K Ambasta and Pravir Kumar (**2015**), *Microbial involvement in cause and treatment of Alzheimer's disease,* 6th World congress on biotechnology, New Delhi, October 5th-7th, *J Biotechnol Biomater* 2015, 5:6

8. **Niraj Kumar Jha**, Saurabh Kumar Jha, Satya Prakash, M. Sonia Angeline, Rashmi K. Ambasta and Pravir Kumar (**2014**), *Physiological stress in neurodegeneration: Interatomic partners based on In silco study,* International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences, 01-03 November, NIMHANS Bangalore, INDIA

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11. Dhiraj Kumar, **Niraj Kumar Jha**, Saurabh Kumar Jha, Renu Sharma, Kushi Anand, Rashmi K. Ambasta and Pravir Kumar (**2014**), *Anti cancerous drugs as a neuroprotectant: a therapeutic intervention in neurodegenerative disorders,* International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences, 01-03 November, NIMHANS Bangalore, INDIA

12. Saurabh Kumar Jha, **Niraj Kumar Jha**, Satya Prakash, M. Sonia Angeline, Rashmi K. Ambasta and Pravir Kumar (**2014**), *In silico* study of flavonones in neurodegenerative disorders, International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences, 01-03 November, NIMHANS Bangalore, INDIA

13. Saurabh kumar Jha, **Niraj kumar Jha**, Rashmi K. Ambasta and Pravir Kumar (**2013**), *Structurally and functionally analyzed new generation anti-aging neuroprotective drugs,* NCRTPSB 2013, JMI 16-18 December, INDIA Journal of Protein and Proteomics, Vol.4, No.2 p62

14. Saurabh kumar Jha, **Niraj kumar Jha**, Deepak Rathore, Rashmi K. Ambasta and Pravir kumar (**2013**), *Traditional FDA approved anti-cancerous drugs versus new anti-cancerous drugs: A computational approach,* 09-11 December, Bioworld Conference, IIT Delhi, Delhi, INDIA

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B. PEER-REVIEWED PUBLICATIONS

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2017

1. Niraj Kumar Jha, Saurabh Kumar Jha, Renu Sharma, Dhiraj Kumar, Rashmi K Ambasta and Pravir Kumar, **(2017)**, Hypoxia induced signaling activation in Neurodegenerative Diseases: Targets for new therapeutic strategies, Under revision ***Journal of Alzheimer's Disease;*** Impact factor: **3.73**
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C. CONFERENCE, PROCEEDINGS AND SYMPOSIUM

2015

1. Niraj Kumar Jha and Pravir Kumar, **(2015)**, Interaction of target drug molecules in hypoxia mediated neurodegeneration, ***29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN)***, December 19-21, 2015, NEHU Shillong **[Oral presentation]**

2. Alka Raina, Saurabh Kumar Jha, Niraj Kumar Jha, Dhiraj kumar, Rashmi K Ambasta and Pravir Kumar, **(2015)**, Putative transcription factor binding elements of ubiquitin E3 ligase in neurodegenerative disorders, ***29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN)***, December 19-21, 2015, NEHU Shillong **[Poster presentation]**

3. Abhisekh Srivastava, Puspendramani Mishra, Dhiraj kumar, Saurabh Kumar Jha, Niraj Kumar Jha, Rashmi K Ambasta and Pravir Kumar, **(2015)**, Relevance of terpenoids and alkaloids in neuroprotection, ***29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN)***, December 19-21, 2015, NEHU Shillong **[Poster presentation]**

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5. Minal Singh, Niraj Kumar Jha, Saurabh Kumar Jha, Dhiraj kumar, Rashmi K Ambasta and Pravir Kumar, **(2015)**, *In silico* characterization of holo AβPP promoter and its transactivation modules, ***29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and***

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7. Pushpendra Mani Mishra, Abhishek Srivastava, Niraj Kumar Jha, Rashmi K Ambasta and Pravir Kumar, **(2015)**, Microbial involvement in cause and treatment of Alzheimer's disease, ***6th World congress on biotechnology***, New Delhi, October 5th-7th, ***J Biotechnol Biomater*** , 5:6 **[Poster presentation]**

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9. Dhiraj Kumar, Niraj Kumar Jha, Saurabh K. Jha, Renu Sharma, Kushi Anand, Rashmi K. Ambasta and Pravir Kumar, **(2014)**, Anti cancerous drugs as a neuroprotectant: a therapeutic intervention in neurodegenerative disorders, ***International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences***, 01-03 November, NIMHANS Bangalore, INDIA **[Poster presentation]**

10. Saurabh Kumar Jha, Niraj Kumar Jha, Satya Prakash, M. Sonia Angeline, Rashmi K. Ambasta and Pravir Kumar, **(2014)**, *In silico* study of flavonones in neurodegenerative disorders, ***International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences***, 01-03 November, NIMHANS Bangalore, INDIA **[Poster presentation]**

2013

11. Niraj kumar Jha, Saurabh kumar Jha, Rashmi K. Ambasta and Pravir kumar, **(2013)** Computer assisted protein analysis in hypoxia signaling, ***NCRTPSB 2013***, JMI 16-18 December, ***INDIA Journal of Protein and Proteomics***, Vol.4, No.2 p37 **[Poster presentation]**

12. Niraj kumar Jha, Lakshmi, Binod Koirala, Saurabh kumar Jha, Renu Sharma, Rohan Kar, Dhiraj, Jitendra Singh, Rashmi K. Ambasta and Pravir kumar, **(2013)**, Identification and validation of key Ubiquitin E3 ligases in type II diabetes: An *in silico* work; 09-11 December, ***Bioworld Conference***, IIT Delhi, Delhi, INDIA **[Poster presentation]**

13. Saurabh kumar Jha, Niraj kumar Jha, Rashmi K. Ambasta and Pravir Kumar, **(2013)**, Structurally and functionally analyzed new generation anti-aging neuroprotective drugs, ***NCRTPSB 2013***, JMI 16-18 December, INDIA ***Journal of Protein and Proteomics***, Vol.4, No.2 p62 **[Poster presentation]**

14. Saurabh kumar Jha, Niraj kumar Jha, Deepak Rathore, Rashmi K. Ambasta and Pravir kumar, **(2013)**, Traditional FDA approved anti-cancerous drugs versus new anti-cancerous drugs: A computational approach, 09-11 December, ***Bioworld Conference***, IIT Delhi, Delhi, INDIA **[Poster presentation]**

2012

15. P. Asthana, A.Sarkar, S. Angeline, Kushi Anand, N. Jaiswal, S.K. Jha, N.K. Jha, Priya Chaterjee R. K. Ambasta, Pravir Kumar, **(2012)**, Protective effect of naringenin on hypoxia induced muscles degeneration. 2012-S-7580-SfN (***Poster presentation in the annual meeting of Society of Neuroscience***, Oct 17, 2012, New Orleans, Poster # 903.16/XX17/F43) USA

D. PROFESSIONAL MEMBERSHIPS

1. Society for Neuroscience, Washington, USA

E. TECHNICAL EXPERIENCE

1. Western Blotting (protein profiling)
2. Confocal Microscopy
3. Polymerase chain reaction (PCR)
4. Paraformaldehyde fixation of tissues
5. Hematoxylin and Eosin staining
6. Immunocytochemistry
7. Competent cell preparation
8. Restriction digestion
9. Cell culture
10. Chick Chorioallantoic Membrane (CAM) Assay
11. Mice model generation
12. Basic bioinformatics tool handling

F. NATIONAL LAEVEL EXAMINATIONS QUALIFIED

1. Qualified **Delhi Technological University PhD written examination & interview in Biotechnology Engg**. 2013 (BT-52)
2. Qualified **DBT- JRF** 2013 (category-B) (Reg.No-12387)
3. Qualified **Jawaharlal Nehru University (JNU) combined entrance** examination 2012 for M.Sc Biotechnology (Rank-GEN-532) (Reg.No-204305)
4. Qualified **Hyderabad Central University (HCU) written** test for "Integrated M.Sc- Ph.D" in Biological Sciences, 2011 (Reg.No-38195063)

G. WORKSHOP AND SEMINARS ATTENDED

1. **Niraj Kumar Jha (2015)**, Work shop on ***advancement in computation Neurochemistry and Neurobiology (SNCI ACNN)***, December 16-18, 2015, NEHU Shillong, **INDIA**

2. Participated in conference on "***Brain and Brain Sciences***: Hot Spot Area in Translational Research" Society for Neurochemistry, India (**SNCI**) VIT University chapter meeting 13th April, 2012, **INDIA**
3. Participated in the special course on "***Plant Transgenic Technology***" jointly organized by the institute of plant genetics, Gottfried Wilhelm Leibniz University, Hannover, Germany and Indo-German Centre for Excellence in Biosciences(IGCEB) in SBST, VIT University 29th January, 2012, **INDIA**
4. Participated in ***"VIT BIO SUMMIT"*** 5th April, **2012** VIT University
5. 5th International Conference on Science, Engineering and Technology (**SET**), VIT UNIVERSITY, 8th November, **2012**, **INDIA**
6. 4th International Conference on Science, Engineering and Technology (**SET**), VIT UNIVERSITY,3th May, **2012**, **INDIA**
7. 3rd International Conference on Science, Engineering and Technology (**SET**), VIT UNIVERSITY, 17th November, **2011**, **INDIA**

H. COMPUTER SKILLS

Conversant with Microsoft Windows and good computer skills with proficiency in common utilities like MS Office, Adobe Photoshop and statistics software (Prism)

I. STRENGTHS

Confidence, Team work spirit & Extrovert personality

J. PERSONAL DETAILS

Date of Birth: 10th December 1990

Languages Known: To Speak: English, Hindi & Maithili

To write: English & Hindi

Age: 27

K. REFERENCES

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Research Article

BIOMOLECULES MEDIATED TARGETING OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN NEURONAL DYSFUNCTION: AN *IN SILICO* APPROACH

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ABSTRACT

Objective: Neurodegenerative diseases are a debilitating age-related disorder manifested by memory loss, impaired motor activity, and loss of muscle tone due to the accumulation of toxic metabolites in the brain. Despite the knowledge of factors causing neurodegenerative disorders, it remains irreversible and incurable. Growing evidence have currently advocated the physiological and pathological contribution of hypoxia-induced vascular endothelial growth factor (VEGF) in neuronal loss. The objective of this research report highlights biomolecules mediated targeting of VEGF activity based on *in silico* approaches that could establish a potential therapeutic window for the treatment of different abnormalities associated with impaired VEGF.

Methods: We employed various *in silico* methods such as drug-likeness parameters, namely, Lipinski filter analysis, Pock Drug tool for active site prediction, AUTODOCK 4.2.1, and LigPlot1.4.5 for molecular docking studies.

Results: Three-dimensional structure of VEGF was generated and Ramachandran plot obtained for quality assessment. RAMPAGE displayed 99.5% of residues in the most favored regions, 0.5% residues in additionally allowed, and no residues in disallowed regions in VEGF, showing that stereochemical quality of protein structure is good. Further, initial screenings of the molecules were done based on Lipinski's rule of five. Finally, we have found Naringenin to be most effective among three biomolecules in modulating VEGF activity based on minimum inhibition constant, Ki, and highest negative free energy of binding with the maximum interacting surface area during docking studies.

Conclusion: The present study outlines the novel potential of biomolecules in regulating VEGF activity for the treatment of different abnormalities associated with impaired VEGF.

Keywords: Hypoxia, Vascular endothelial growth factor, Biomolecules, Active site prediction, Molecular docking.

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INTRODUCTION

Neurodegenerative diseases are pathological conditions that have an insidious onset and chronic progression. Different models have been established to study these diseases to understand their underlying mechanisms and to investigate new therapeutic strategies [1]. Several downstream signaling molecules are reported to trigger under hypoxia. Vascular endothelial growth factor (VEGF) is one of them, which is responsible for the formation of new blood vessels (angiogenesis) and lead to the supply of nutrients and oxygen for normal homeostasis [2]. Moreover, the crucial role of VEGF in the brain is not restricted only to controlling vessel growth: But it has direct effects on different types of neural cells including neural stem cells. Conversely, altered expression of this molecule has been implicated in virtually every type of angiogenic disorder, including those associated with cancer, ischemia, and inflammation [3]. Moreover, studies have also revealed the pathological implication of VEGF in the progression of neurodegenerative disorders (NDDs) including, Alzheimer's disease, Huntington disease, and amyotrophic lateral sclerosis. Recent genetic studies have revealed that reduced VEGF levels cause neurodegeneneration through impairing neural tissue perfusion [4]. Importantly, implementation of different biomolecules may helpful in regulating the altered levels of VEGF in cells. The growing evidence for an etiologic role of VEGF in neurodegeneration provides an underlying principle for considering the therapeutic potential of VEGF for NDDs, which are mostly not curable. In this framework, we have introduced different biomolecules (naringenin, quercetin, and sesamol) for targeting VEGF. These biomolecules have chemoprotective, anti-inflammatory, neuroprotective, and anti-aging property. For this purpose, we have performed *in silico* based structural and functional analysis of these molecules for revealing its therapeutic importance against neuronal loss through modulating the impaired expression of VEGF. The objective of this study is to explore neuroprotective action of these biomolecules in regulating the altered level of VEGF to attenuate the toxicity associated with toxic proteins in neuronal death.

METHODS

Visualization and quality assessment of three-dimensional (3D)-structure of VEGF

3D-structure of VEGF (ID: 1QTY) was generated using protein data bank (PDB), structural evaluation, and stereochemical analysis was performed using RAMPAGE (http://www.mordred.bioc.cam.ac.uk/~rapper/rampage.php). Errat server was used to find the accuracy of the structure and visualization of determined structures was performed using University of California, San Francisco Chimera.

Active site prediction

The active sites of VEGF were predicted using the Pock Drug tool (http://pockdrug.rpbs.univ-paris-diderot.fr/cgi-bin/index.py?page=home). The PDB structure of VEGF was uploaded and active sites were predicted using f-pocket estimation and setting ligand proximity threshold at 5.5.

Ligand optimization

Reported ligand molecules along with their physical and chemical properties were retrieved from PubChem Compound Database (http://

www.pubchem.ncbi.nlm.nih.gov/). PubChem is a composite database that is backed up by three primary databases, i.e., polycarbonate compounds (PC) substance, PC compund, and PC BioAssay. PubChem provides biological activity and chemical information of small molecules. PC substance contains information about the substances; PC compound contains information about chemical compounds, and PC BioAssay provides information about Bioassays. Three compounds (naringenin, quercetin, and sesamol) were selected. SDF files of ligands were converted in PDB file with the help of Open Babel tool that could be used for docking study. Visualization of molecular structure of compounds was done using PyMOL.

Lipinski filter analysis of screened drugs

An online tool Lipinski filter (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp) was used to retrieve the information about drug-likeness properties of biomolecules with the help of Lipinski rule of five. Lipinski rule helps differentiate drug and non-drug such as properties of molecules. It is used to identify the possibility of success or failure due to drug-likeness for molecules fulfiling with two or more of the following rules: (a) Molecular mass should be <500 Da, (b) high lipophilicity (expressed as log p<5), (c) <5 hydrogen bond donors, (d) <10 hydrogen bond acceptors, and (e) molar refractivity should be between 40 and 130.

Preparation of protein and ligand molecules

Preparation of protein involves the addition of polar hydrogen atoms, neutralization of charge, and removal of any miscellaneous structures from the protein molecule by AUTODOCK 4.2.1 whereas ligand preparation involves the neutralization of charge.

Molecular docking studies

Prepared and optimized structures of ligands and protein were ultimately used for molecular docking using AUTODOCK 4.2.1 for predicting the possible protein-ligand interactions and the results that include the understanding of the association that involves H-bonding, and hydrophobic interactions were analyzed using LIGPLOT1.4.5, a program to generate schematic diagrams of protein-ligand interactions.

RESULTS

3D-structure visualization and quality assessment

3D-structure of VEGF was generated and visualized using UCSF Chimera (Fig. 1a). Even though, there were no steric clashes in the structure generated, it was assessed for geometric and energy aspects. Ramachandran plot was used to check the reliability of predicted 3D-structure of VEGF. RAMPAGE checks the stereochemical quality of a protein structure by analyzing residue-by-residue geometry and overall structural geometry. Ramachandran plot was obtained for VEGF for quality assessment. RAMPAGE displayed 99.5% of residues in the most favored regions, 0.5% residues in additionally allowed, and no residues in disallowed regions in VEGF (Fig. 1b). Errat server was used to determine the accuracy of the model. Result of Errat showed 95.694% accurate structure for VEGF.

Active site prediction

Of top 10 pockets, VEGF had best pocket at P4 with a drug ability score of 0.95 and 0.01 standard deviation (Table 1). The volume of given pocket was 1338.57 cubic angstroms and fourteen residues were involved in interaction at this site.

Lipinski filter analysis of screened drugs

Further, the screening of ligand molecules was done on the basis of Lipinski's rule of five. Lipinski filter analysis revealed that all the compounds selected possessed drug likeness and can be used for docking purposes (Fig. 2).

Molecular docking of VEGF with biomolecules

Biomolecules bound to VEGF at P4 pocket and same residues as predicted were involved in the interaction. The estimated free energy of binding for VEGF and naringenin was - 7.56 kcal/mol and total intermolecular energy was - 9.05 kcal/mol. Similarly, the estimated free energy of binding for VEGF and quercetin was - 7.10 kcal/mol and total intermolecular energy was - 8.89 kcal/mol. Likewise, the estimated free energy of binding for VEGF and sesamol was - 5.09 kcal/mol and total intermolecular energy was - 5.39 kcal/mol. Molecular docking pattern of VEGF with screened molecules (naringenin, quercetin, and sesamol) have been identified and depicted in Fig. 3. On the basis of docking analysis, interacting compounds with minimum binding constant and highest negative free energy of binding are most effective. Docking calculation of VEGF with these molecules has been presented in Table 2.

Binding site of VEGF with selected compounds along with its reported Inhibitory active site

Binding site residues of VEGF interacting with naringenin, quercetin, and sesamol were found to be the same as the residues involved in their respective catalytic sites. Interacting residues of VEGF with naringenin, quercetin, and sesamol along with their identified catalytic sites have

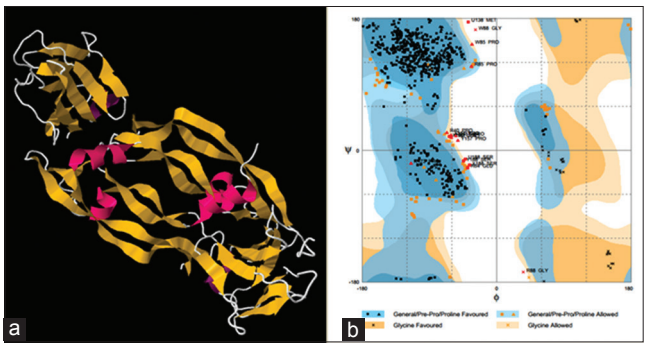

Fig. 1: (a) Three-dimensional-structure and (b) Ramachandran plot of vascular endothelial growth factor protein

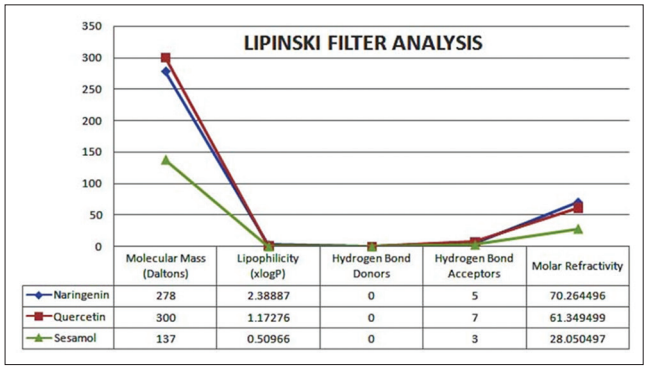

Fig. 2: Differentiation of drugs on the basis of Lipinski rule of five by Lipinski filter

Table 1: Active sites of vascular endothelial growth factor AQ4

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	2205.45	-0.58	0.67	0.2	0.0	30.0	0.63	0.09
P 1	2500.5	-0.67	0.66	0.19	0.0	32.0	0.59	0.08
P 16	452.91	0.83	0.43	0.0	0.0	14.0	0.93	0.02
P 18	744.97	0.56	0.5	0.14	0.0	14.0	0.94	0.01
P 19	455.66	0.83	0.43	0.0	0.0	14.0	0.93	0.02
P 2	1622.2	-0.86	0.68	0.24	0.0	25.0	0.49	0.12
P 3	1819.24	-0.87	0.69	0.21	0.03	29.0	0.53	0.03
P 4	1338.57	0.34	0.62	0.19	0.04	21.0	0.95	0.01
P 5	1159.82	-0.3	0.67	0.24	0.04	21.0	0.83	0.02
P 6	1368.17	-1.2	0.75	0.06	0.0	16.0	0.14	0.02

been show in Table 3 and their two-dimensional and 3D pattern of interaction is presented in Fig. 4.

DISCUSSION

Neurodegeneration is an umbrella term for a range of conditions which primarily affect the neurons in the human brain [5]. Despite the knowledge of various factors which contribute in the occurrence and progression of NDDs, the exact cause and cure remains elusive. Abnormal expression of VEGF protein in terminally differentiated neurons is a recently known phenomenon which has been shown to drive neurodegeneration followed by apoptosis [6]. Free-radical injury of microvessels under hypoxia causes neuroinflammation and oligemia which thereafter leads to Aβ accumulation through vascular damage and the activation of proangiogenic factors including, hypoxia-inducible factor-1α, and VEGF-1 [7]. Further, invading macrophages and monocytes also causes neuronal damage through activation of VEGF-1 [8]. Similarly, low VEGF levels not only impair spinal cord perfusion and cause chronic ischemia of motoneurons but also deprive these cells of vital VEGF-dependent survival and neuroprotective signals.

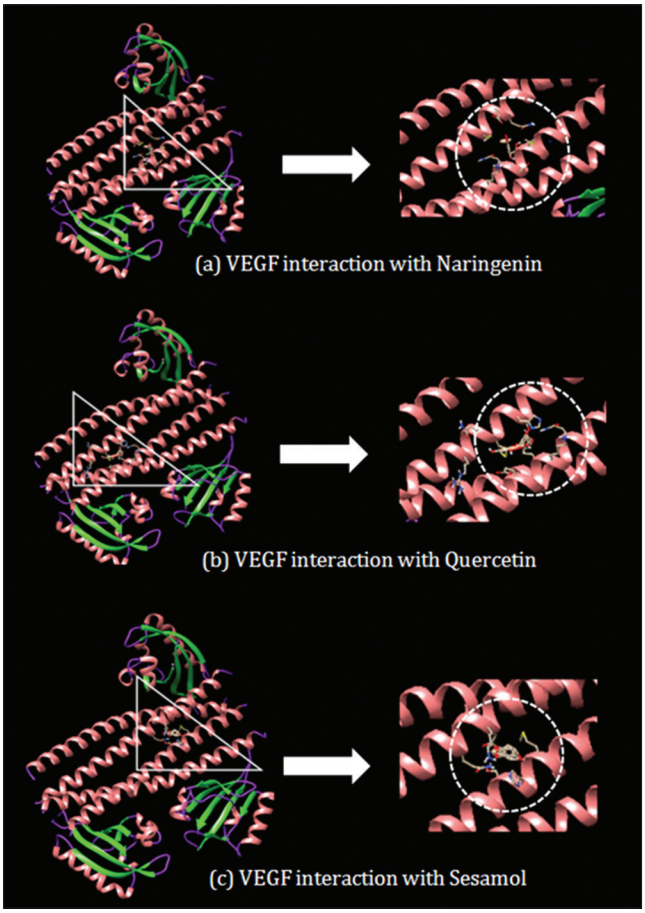

Fig. 3: Binding of vascular endothelial growth factor with selected compounds

Both phenomenons result in progressive degeneration of motoneurons, associated with muscle weakness, paralysis, and death [9]. Thus, it seems imperative to design therapeutic strategies aimed at attenuating the altered level of VEGF to inhibit the cascade of neurodegeneration.

Flavonoids have been advocated to exert human health benefits by antioxidant and anti-inflammatory mechanisms [10]. Naringenin reportedly prevent oxidative stress and nuclear factor kappa B (NF-κB)-mediated inflammatory brain damage in the rat model of focal cerebral injury. Further, prophylactic treatment with naringenin ameliorated functional outcomes and abrogated the ischemic brain injury by suppressing NF-κB-mediated neuroinflammation [11]. Similarly, a significant raise in neuronal survivability was observed with quercetin treatment in rats administered 6-OHDA. Both naringenin and quercetin also reversed the effect of hypobaric hypoxia and elicit neuroprotective response by reducing VEGF level in the murine model [12]. Further, sesamol pre-treatment restored oxidative defense possibly by its free radical scavenging lighted the neuroprotective effect of sesamol against 3-NP-induced neuronal damage [13]. Taken together, all these data provide convincing evidence of using VEGF interaction bioflavonoids such as naringenin and quercetin in attenuating the level of VEGF and in turn, inhibit the cascade of neuronal death.

RAMPAGE displayed 99.5% of residues in the most favored regions, 0.5% residues in additionally allowed and no residues in disallowed regions in VEGF, showing that stereochemical quality of protein structure is good. Result of Errat showed 95.694% accurate structure for VEGF. Lipinski filter analysis of all the compounds revealed that these compounds could act such as a drug and have drug-like property as these compounds meet the criteria of Lipinski Rule of five. Finally, molecular docking studies indicated that all these compounds can bind to and modulate the level of VEGF and possibly, halt or inhibit toxic proteins induced neuronal death in NDDs. Docking study revealed that all three compounds are interacting at the reported active binding site and binding atomic coordination was compared with the template complex coordination and found that docked drug coordination was similar with the known coordination. Amino acid residues of VEGF involved in interaction with naringenin, quercetin, and sesamol were found to be the same as the residues involved in binding with earlier used inhibitors. These observations clearly indicate that we can efficiently determine active site coordinates to investigate the effect of inhibitors on the functional active site of protein. In this result, the most effective compound was found to be naringenin as showing minimum inhibition constant, Ki, and lowest free energy of binding with maximum interacting surface area [14-17]. These findings can be further validated through *in vitro* and *in vivo* studies in neurodegeneration. Overall, although further work is required, these studies advocate the pivotal role of VEGF in NDDs and provide adequate grounds for estimating the potential therapeutic effectiveness of VEGF in their management.

CONCLUSION

The results of our study provide novel potential of biomolecules such as naringenin, quercetin, and sesamol in regulating VEGF expression in the brain, which has wider implications in the progression as well as protection against NDDs. Moreover, of these three biomolecules naringenin is showing better interaction with VEGF based on their minimum binding constant and highest negative free energy.

Table 2: Docking calculation of compounds with VEGF

Compound name	Estimated free energy of binding (kcal/mol)	Estimated binding constant (μM)	Estimated intermolecular energy (kcal/mol)	vdW+Hbond+desolv energy (kcal/mol)	Electrostatic energy (kcal/mol)	Estimated internal energy (kcal/mol)	Torsional free energy (kcal/mol)
Naringenin	−7.56	1.74	−9.05	−9.03	−0.02	+9.69	+1.19
Quercetin	−7.10	6.21	−8.89	−8.73	−0.16	+9.49	+1.79
Sesamol	−5.09	186.16	−5.39	−5.33	−0.06	+0.33	+0.30

VEGF: Vascular endothelial growth factor

Table 3: VEGF known inhibitory site and selected compounds interacting residues

Compounds	Interacting residues
Reported active site	PHE^{375}, VAL^{376}, ASP^{377}, HIS^{378}, ARG^{379}, VAL^{381}, ALA^{382}, GLY^{385}, GLN^{387}, PRO^{388}, GLN^{389}, GLU^{390}, LEU^{392}, LYS^{432} and ASN^{433} of chain A. GLN^{434}, MET^{605}, THR^{606}, GLU^{608}, GLN^{609}, LYS^{610}, LYS^{612}, GLU^{613}, and GLU^{616} of chain B. MET^{598}, HIS^{601}, SER^{602}, MET^{605}, THR^{606}, LEU^{607}, GLU^{608}, GNL^{609}, THR^{611}, LYS^{612}, GLU^{613}, ILE^{614}, ASP^{615} and GLU^{616} of chain C. ARG^{594}, MET^{598}, HIS^{601}, SER^{602}, MET^{605}, THR^{606}, GLN^{609}, LYS^{612} and GLU^{613} of chain F. SER^{241}, LYS^{242}, ASP^{259}, ARG^{260}, THR^{262}, GLU^{263}, LEU^{264}, ILE^{265}, GLY^{266}, HIS^{268}, PRO^{269}, GLU^{270}, ALA^{311}, LYS^{312}, HIS^{313}, GLY^{314}, GLY^{315}, TYR^{316}, VAL^{317}, TRP^{318}, VAL^{343}, and LEU^{344} of Chain G
Naringenin	MET^{605}, THR^{606}, GLU^{608}, and GLN^{609} residues of chain C and MET^{605}, THR^{606}, and GLN^{609} residues of chain F
Quercetin	GLN^{609}, LYS^{612}, GLU^{613}, and GLU^{616} residues of chain C and ARG^{594}, MET^{598}, HIS^{601}, and SER^{602} residues of chain F
Sesamol	MET^{598}, HIS^{601}, and SER^{602} residues of chain C and GLN^{609}, LYS^{612}, and GLU^{613} residues of chain F

VEGF: Vascular endothelial growth factor

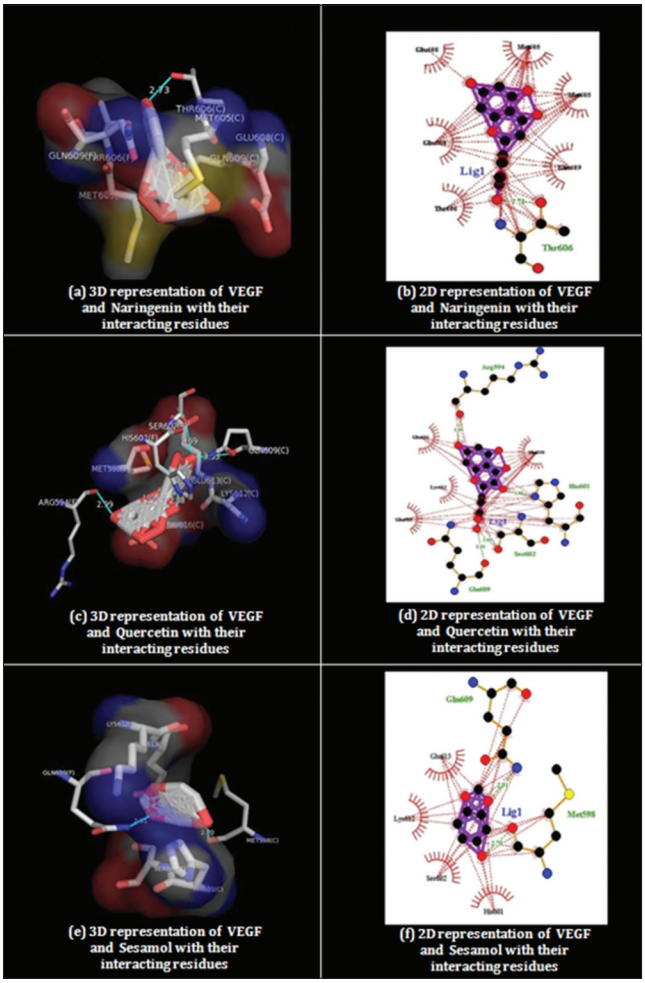

Fig. 4: Three-dimensional- and two-dimensional-representation of vascular endothelial growth factor and ligand interaction

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Author Queries???

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Original Article

MOLECULAR DOCKING STUDIES FOR THE COMPARATIVE ANALYSIS OF DIFFERENT BIOMOLECULES TO TARGET HYPOXIA INDUCIBLE FACTOR-1α

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ABSTRACT

Objective: Hypoxia plays a significant role in governing many vital signalling molecules in the central nervous system (CNS). Hypoxic exposure has also been depicted as a stimulus for oxidative stress, increase in lipid peroxidation, DNA damage, blood-brain dysfunction, impaired calcium (Ca^{2+}) homoeostasis and agglomeration of oxidized biomolecules in neurons, which act as a novel signature in diverse neurodegenerative and oncogenic processes. On the contrary, the presence of abnormally impaired expression of HIF-1α under hypoxic insult could serve as an indication of the existence of tumors and neuronal dysfunction as well. For instance, under hypoxic stress, amyloid-β protein precursor (AβPP) cleavage is triggered due to the higher expression of HIF-1α and thus leads to synaptic loss. The objective of this research is to perform comparative studies of biomolecules in regulating HIF-1α activity based on *in silico* approaches that could establish a potential therapeutic window for the treatment of different abnormalities associated with impaired HIF-1α.

Methods: We employed various *in silico* methods such as drug-likeness parameters namely Lipinski filter analysis, Muscle tool, SWISS-MODEL, active site prediction, Auto Dock 4.2.1 and LigPlot1.4.5for molecular docking studies.

Results: 3D structure of HIF-1α was generated and Ramachandran plot obtained for quality assessment. RAMPAGE displayed 99.5% of residues in the most favoured regions. 0% residues in additionally allowed and 0.5% disallowed regions of the HIF-1α protein. Further, initial screenings of the molecules were done based on Lipinski's rule of five. Cast P server used to predict the ligand binding site suggests that this protein can be utilised as a potential drug target. Finally, we have found *Naringenin* to be most effective amongst three biomolecules in modulating HIF-1α based on minimum inhibition constant, Ki and highest negative free energy of binding with the maximum interacting surface area during docking studies.

Conclusion: The present study outlines the novel potential of Biomolecules in regulating HIF-1α activity for the treatment of different abnormalities associated with impaired HIF-1α.

Keywords: Hypoxia-inducible factor-1α (HIF-1α), Biomolecules, Active site prediction, Molecular docking

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INTRODUCTION

Hypoxia plays a decisive role in controlling many important signalling molecules in the central nervous system (CNS) [1]. At the higher altitude, low barometric pressure causes accelerated expression of HIF gene. Hypoxia inhibits prolyl hydroxylation of HIF-1α leading to aggregation of a functional heterodimeric transcription factor (both HIF1 α and HIF1 β subunits) [2]. Hypoxia-inducible factor-1 (HIF-1) is a key transcriptional factor which is amenable for cellular adaption to low oxygen tension. It is a heterodimer comprising of an oxygen-regulated α-subunit and a constitutively expressed β-subunit that regulates a series of genes associated with iron metabolism, angiogenesis, cell proliferation/survival and glucose metabolism [3].

The activity of HIF-1 is regulated by post-translational modifications on different amino acid residues of its subunits, mainly the α-subunit. It has been reported that under hypoxic insult, the activity of HIF-1 and expression of its associated downstream genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO) are altered in a range of neurodegenerative diseases (NDDs) including Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS) [4]. It has also been reported that hypoxia causes reduced synaptic transmission associated with neuronal death by causing neuronal dysfunction [5]. Thus, elucidation of pathophysiological mechanisms caused due to hypoxic insults on the cerebral nervous system (CNS); their therapeutic regulation awaits much importance. Further, experimental and clinical evidence has revealed that regulating the expression of HIF-1α might improve the cellular and tissue damage in the NDDs. This regulatory role on the expression of HIF-1α can be accompanied by employing different biomolecules.

Naringenin (5, 7, 4-trihydroxyflavanone, NGEN) is a flavanone, mainly found in the citrus fruits and tomato. It is known to be act as a multi-functional agent. For example, it acts as a powerful anti-oxidant, anti-depressant, anti-inflammatory and neuroprotective compound [6]. Similarly, *Sesamol* is the major constituent of sesame seed oil (*Sesamum indicum*) possessing powerful antioxidant property. Further, it acts as chemoprotective, anti-inflammatory, neuroprotective, hepatoprotective, and anti-aging biomolecule [7]. Another, well-known biomolecules, *Quercetin* (2-(3,4-dihydroxyphenyl)-3,5, 7-tryhydroxy-4H-chromen-4-one, QUR) possess anti-oxidant, anti-inflammatory and in some cases anti-cancerous activities [8]. These biomolecules are having neuroprotective properties as proposed to be potent therapeutic agents in many diseases, including cognitive impairment associated with neuronal damage. Although, regulating the function of HIF-1α are crucial to be investigated that have protective effects on the pathological ambiances resulting from hypoxic insults in the brain. Therefore, in this research report, we have done *in silico* based comparative study of three different biomolecules (*Naringenin, Sesamol* and *Quercetin*) in order to regulate the impaired functions of HIF-1α. These molecules have been identified and selected for this study through Lipinski rule of five. However, several *in vitro* studies have explored its neuroprotective activities in different neuronal cell cultures and animal models [9, 10]. But a comparative study of these biomolecules on the expression of HIF-1α by using *in silico* tools has not been explored so far, which we are going to explore in this article. In the present study, we reported the Phylogenetic and Physico-chemical properties of HIF-1α gene, since we have selected this gene as a target protein for docking study. Further, Homology modelling visualization and quality assessment of 3D-structure of HIF-1α has been addressed. Additionally, active site prediction and ligand optimisation for both the biomolecules and protein has been done in order to perform molecular docking. Further, we found and docking study revealed that all three compounds were interacting at the reported active and binding site. Finally, in this result the most effective compound was found to be *Naringenin* as showing

minimum Inhibition Constant, Ki and highest negative free energy of binding with maximum interacting surface area suggesting that *Naringenin* could be effective as HIF-1α regulators and therefore act as potential therapeutic molecules for treating patients suffering from hypoxic insults.

MATERIALS AND METHODS

Retrieval of hypoxia inducible factor protein and its function recognition

The amino acid sequence of hypoxia inducible factor protein HIF-1α with accession number Q16665.1 of *Homo sapiens* was retrieved from NCBI database and was used for homology search using Basic Local Alignment Search Tool (BLAST). Protein functional elucidation was done using Interproscan server (https://www.ebi.ac.uk/interpro/search/sequence-search).

Phylogenetic relationship and physicochemical properties

For multiple sequence analysis Muscle tool (http://www.ebi.ac.uk/Tools/msa/muscle/) was used and a phylogenetic tree was constructed using Muscle tool based on NJ (Neighbor-joining) plot without distance correction. ProtParam (http://web.expasy.org/protparam/) was used to predict physicochemical properties. The parameters computed by ProtParam included the molecular weight, theoretical PI, aliphatic index and grand average of hydropathicity (GRAVY).

Homology modelling, visualisation and quality assessment of 3D-structure of hypoxia-inducible factor

Homology modeling was used to determine the 3D-structure of HIF-1αisoforms. A BLASTP search with default parameters was performed against the Brookhaven Protein Data Bank (PDB) to find suitable templates for homology modelling. Template with PDB ID: 4H6J was retrieved for HIF-1αprotein from PDB. The Protein Structure Prediction Server SWISS-MODEL (http://swissmodel.expasy.org/) was used for homology model construction. Once the 3D-structure of proteins was generated, structural evaluation and stereochemical analysis were performed using RAMPAGE (http://www.mordred.bioc.cam.ac.uk/~rapper/rampage.php). Errat server was used to find the accuracy of the structure and visualisation of determined structures was performed using UCSF Chimera.

Active site prediction

Castp Server (http://www.sts.bioe.uic.edu/castp/) was used to predict the active sites of the protein. Castp could also be used to measure area, the circumference of mouth openings of each binding site insolvent and molecular accessible surface. PDB file of protein was uploaded in the server and it showed the ligand binding sites present in protein and the site with maximum surface area and maximum surface volume was selected and all the amino acid residues involved in binding with ligands were retrieved.

Ligand optimization

Reported ligand molecules along with their physical and chemical properties were retrieved from Pub chem compound database (http://www.pubchem.ncbi.nlm.nih.gov/). Pubchem is a composite database that is backed up by three primary databases, i.e. PC substance, PC compound, and PC BioAssay. Pubchem provides biological activity and chemical information of small molecules. PC substance contains information about the substances; PC compound contains information about chemical compounds, and PCBio assay provides information about Bioassays. Four compounds (*Naringenin, Quercetin,* and *Sesamol*) were selected. SDF files of Ligands were converted in PDB file with the help of Open Babel tool that could be used for docking study. Visualization of Molecular Structure of compounds was done using Pymol.

Lipinski filter analysis of screened drugs

An online tool Lipinski Filter (http://www.scfbio-iitd.res.in/software/drug design/lipinski.jsp) was used to retrieve the information about drug-likeness properties of Biomolecules with the help of Lipinski rule of five. Lipinski rule helps to differentiate drug and non-drug like properties of molecules. It is used to identify the possibility of success or failure due to drug-likeness for molecules fulfilling with two or more of the following rules: (a) Molecular Mass should be less than 500 Dalton, (b) High Lipophilicity (expressed as logP less than 5), (c) Less than 5 hydrogen bond donors, (d) Less than 10 hydrogen bond acceptors and (e) Molar refractivity should be between 40-130.

Preparation of protein and ligand molecules

Preparation of protein involves the addition of polar hydrogen atoms, neutralisation of charge and removal of any miscellaneous structures from the protein molecule by Autodock 4.2.1 whereas ligand preparation involves the neutralization of charge.

Molecular docking studies

Prepared and optimised structures of ligands and protein were ultimately used for molecular docking using Autodock 4.2.1 for predicting the possible protein–ligand interactions and the results that include the understanding of the association that involves H-bonding and hydrophobic interactions were analyzed using LigPlot1.4.5, a program to generate schematic diagrams of protein-ligand interactions.

RESULTS

Retrieval of hypoxia inducible factor protein and its functional elucidation

Based on functional domain sequence of well-characterized gene/protein, a homology search was done using BLAST. We have successfully hunted 5 isoforms (table 1) of protein HIF-1α on the basis of families and domains identified from Interproscan results. Interproscan study revealed that all homologues proteins for HIF-1α were belonging to Hypoxia-inducible factor, α-subunit family (IPR021537), Hypoxia-inducible factor-1αfamily (IPR001321), Myc-type, basic helix-loop-helix domain (IPR011598), PAS domain (IPR000014), PAS fold (IPR013767), PAS fold 3 (IPR013655), HIF-1α, transactivation domain, C-terminal (IPR014887) and a repeat of PAC motif (IPR001610)respectively (fig. 1).

Table 1: Hunted HIF-1α related proteins

S. No.	Accession No.	Protein	Score	Identity	E Value
1	NP_001521.1	hypoxia-inducible factor 1-alpha isoform 1	1721	100%	0
2	AKI70676.1	HIF1A	1719	99%	0
3	AAC68568.1	hypoxia-inducible factor 1 alpha subunit	1718	99%	0
4	NP_001230013.1	hypoxia-inducible factor 1-alpha isoform 3	1696	99%	0
5	Q9XTA5.1	Hypoxia-inducible factor 1-alpha	1624	95%	0

Phylogenetic relationship and physicochemical properties

For multiple sequence analysis, Muscle tool was used and found that amino acid residues were conserved in most of the isoforms of the protein HIF-1α (fig. 2a). A phylogenetic study of HIF-1αhunted proteins revealed that HIF-1α and HIF1A were in the same cluster as they share the same homology and HIF-1α isoform 1 was in another cluster while HIF-1α isoform 3 and HIF-1α subunit were differed from others (fig. 2b). ProtParam showed that Mol. wt. of HIF-1α was 92670.4 Daltons. An isoelectric point for HIF-1αwas 5.17 which indicates that protein was negatively charged. The GRAVY index of-0.573 for HIF-1α is indicative of hydrophilic and soluble protein (table 2).

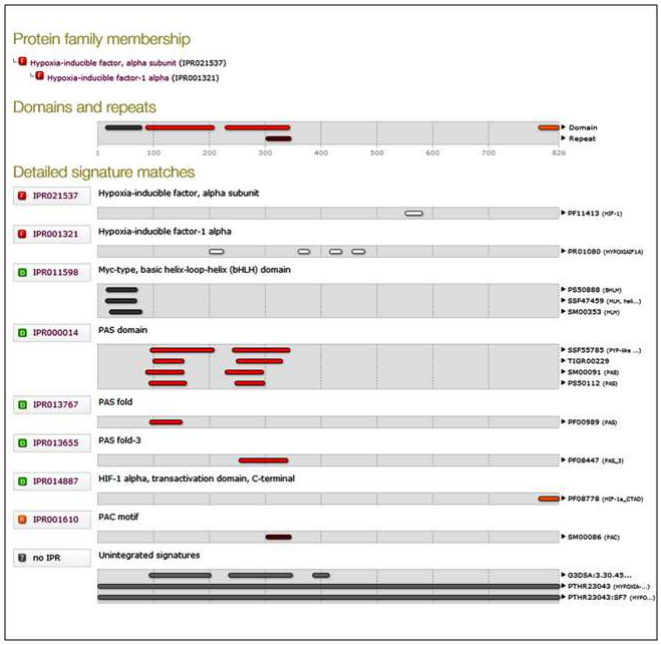


Fig. 1: Interproscan result for HIF-1-αfamily and their domain identification

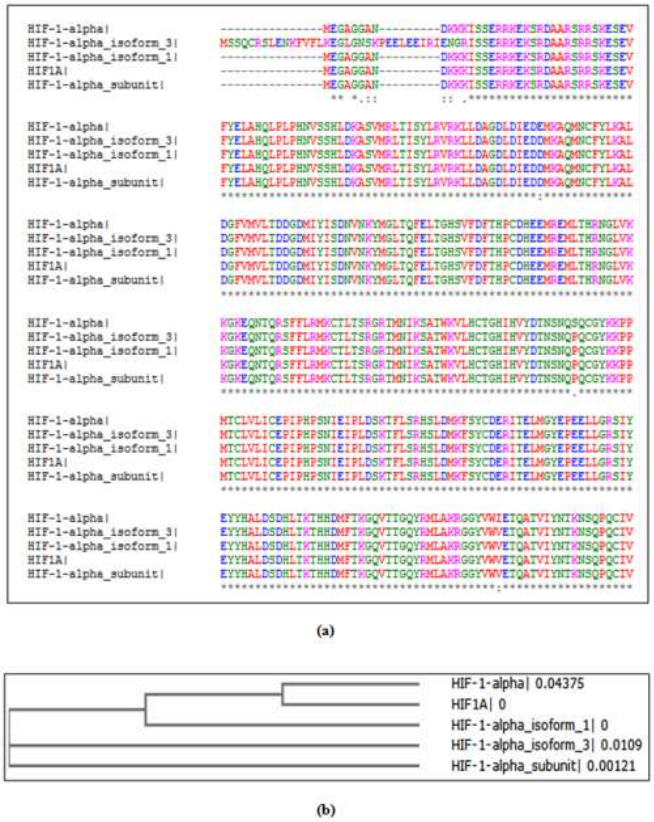


Fig. 2: (a) Multiple sequence alignment and (b) phylogenetic analysis of all HIF-1αisoforms

Table 2: Physico-chemical properties of HIF-1α

Properties	HIF-1α
Molecular Formula	$C_{4027}H_{6410}N_{1108}O_{1309}S_{43}$
Molecular Weight (Daltons)	92670.4
Theoretical PI	5.17
Aliphatic Index	74.96
Grand Average of Hydropathicity (GRAVY)	-0.573

Homology modelling

Prediction of 3D-structure of proteins provides us precise functional information of how proteins interact and localize in their stable conformation. Homology modelling is a most common structure prediction method in structural genomics and proteomics. The best matching template was selected for the target protein on the basis of sequence homology using PDB Advance Blast. The template is experimentally determined 3D-structure of protein that shares sequence similarity with the target sequence. Template showed a sequence identity of 99.07% for HIF-1αisoforms. 3D-structure of HIF-1αwas generated using Swiss-Model Server. The Z-score is indicative of overall model quality and is used to check whether the input structure is within the range of scores typically found for native proteins of similar size. Z-score of the template and query model was obtained by SWISS-MODEL. Z-score for HIF-1α was-0.81 suggesting a good structure (table 3).

Table 3: Swiss model server result showing template structure used in homology modelling, sequence identity and quality score of the model generated

Gene name	Modelled residue range	Based on template	Sequence identity	QMEAN Z-score
HIF-1α	139-811	4H6J	99.07%	-0.81

3D-structure visualization and quality assessment

3D-structure of HIF-1α transcription factor was generated and visualized using UCSF Chimera (fig. 3a). Even though there were no steric clashes in the structure generated, it was assessed for geometric and energy aspects. Ramachandran plot was used to check the reliability of predicted 3D-structure of hypoxia inducible factor protein HIF-1α. RAMPAGE checks the stereochemical quality of a protein structure by analysing residue-by-residue geometry and overall structural geometry. Ramachandran plot was obtained for HIF-1αfor quality assessment. RAMPAGE displayed 99.5% of residues in the most favoured regions, 0.5% residues in additionally allowed and no residues in disallowed regions in HIF-1αProtein (fig. 3b). Errat server was used to determine the accuracy of the model. The result of Errat showed 95.694% accurate structure for HIF-1α protein.

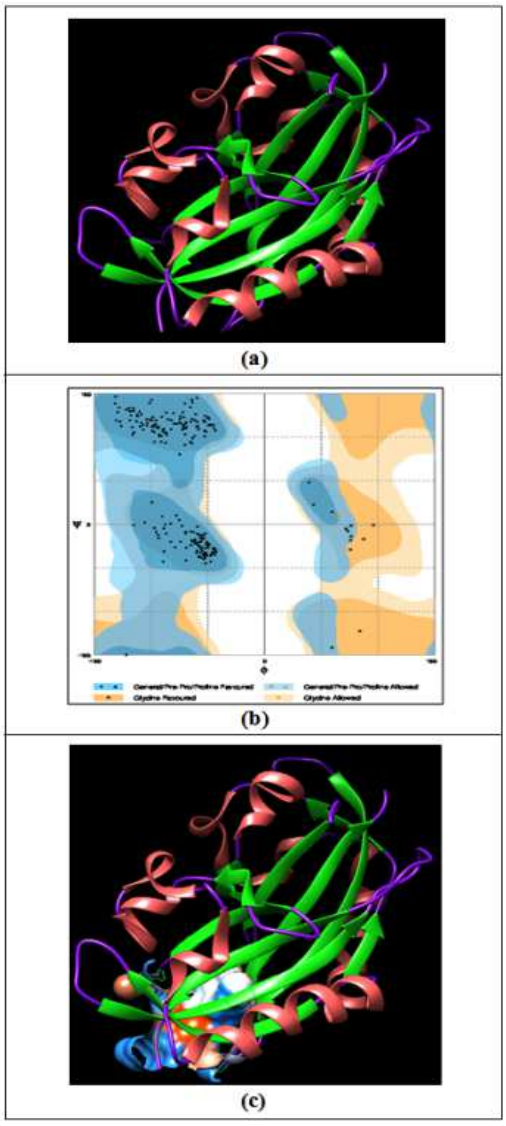
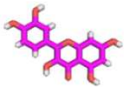
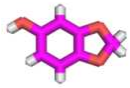


Fig. 3: (a) 3D-Structure, (b) Ramachandran Plot and (c) Active site of generated HIF-1α model

Active site prediction

CastP server was used to predict the ligand binding sites in the generated 3D-structure of HIF-1α. This server calculates the possible active sites from the 3D atomic coordinates of the protein (fig. 3c). Among the twenty-nine binding sites obtained from CastP for HIF-1α, site 29 was highly conserved within the active site of the protein. The Predicted site 29 consisted 415.4 Cubic angstroms site volume out of the 1661.1 Cubic Angstroms of protein volume. The residues in site twenty-nine are shown in (table 6).

Table 4: Physico-chemical properties of natural compounds used for docking study

Characteristics	Naringenin	Quercetin	Sesamol
Molecular weight	272.25278 g/mol	302.2357 g/mol	138.12074 g/mol
Molecular Formula	$C_{15}H_{12}O_5$	$C_{15}H_{10}O_7$	$C_7H_6O_3$
Molecular Structure			
IUPAC Name	5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	1,3-benzodioxol-5-ol
Rotatable Bond Count	1	1	0
Topological Polar Surface Area	$87A^2$	$127A^2$	$38.7A^2$
Heavy Atom Count	20	22	10
Complexity	363	488	126

Further, the screening of ligand molecules was done on the basis of Lipinski's rule of five. Lipinski filter analysis revealed that all the compounds selected possessed drug likeness and can be used for docking purposes (fig. 4).

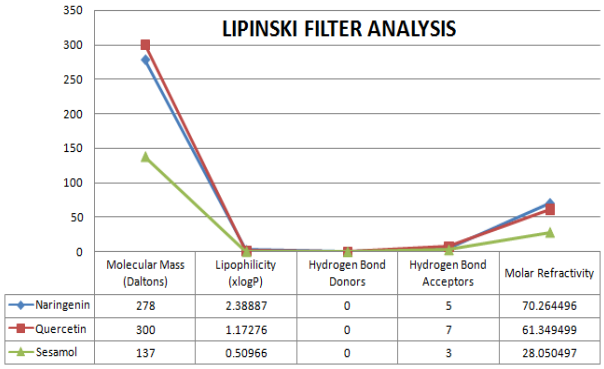


Fig. 4: Differentiation of drugs on the basis of lipinski rule of five by lipinski filter

Docking calculation of compounds with HIF-1A

HIF-1α interaction with *Naringenin*

Free energy of binding with *Naringenin*was-8.48 kcal/mol and Est. Inhibition Constant, Ki was found to be 605.40nM. Intermolecular Energy was found to be-9.68 kcal/mol. VdW+Hbond+desolv Energy and Electrostatic Energy was-9.62 kcal/mol and-0.06 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be 9.70 kcal/mol and 1.19 kcal/mol.

HIF-1α interaction with *Quercetin*

Free energy of binding with *Quercetin*was-8.22 kcal/mol and Est. Inhibition Constant, Ki was found to be 945.18 nM. Intermolecular Energy was found to be-10.01 kcal/mol. VdW+Hbond+desolv Energy and Electrostatic Energy was-9.81 kcal/mol and-0.20 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be 9.51 kcal/mol and 1.79 kcal/mol.

HIF-1α interaction with *Sesamol*

Free energy of binding with *Sesamol* was-5.13 kcal/mol and Est. Inhibition Constant, Ki was found to be 174.23 µM. Intermolecular Energy was found to be-5.43 kcal/mol. VdW+Hbond+desolv Energy and Electrostatic Energy was-5.34 kcal/mol and-0.08 kcal/mol. Total Internal Energy and Torsional Free Energy was found to be 0.32 kcal/mol and 0.30 kcal/mol. Docked energy estimation of HIF-1α is shown in table 5 and interaction of HIF-1α with ligands is shown in (fig. 5).

Binding site of HIF-1α with selected compounds along with its reported Inhibitory active site

Binding site residues of HIF-1α interacting with *Naringenin, Quercetin* and *Sesamol* were found to be the same as the residues involved in their respective catalytic sites. Interacting residues of HIF-1α with *Naringenin, Quercetin* and *Sesamol* along with their identified catalytic sites have been shown in (table **6**) and their 2D and 3D pattern of interaction is presented in (fig. 6).

Table 5: Docking calculation of compounds with HIF-1α

Compound name	Est. free energy of binding	Est. binding constant	Est. intermolecular energy	vdW+Hbond+desolv energy	Electrostatic energy	Est. internal energy	Torsional free energy
Naringenin	-8.48 (kcal/mol)	605.40 nM	-9.68 (kcal/mol)	-9.62 (kcal/mol)	-0.06 (kcal/mol)	+9.70 (kcal/mol)	+1.19 (kcal/mol)
Quercetin	-8.22 (kcal/mol)	945.18 nM	-10.01(kcal/mol)	-9.81 (kcal/mol)	-0.20 (kcal/mol)	+9.51 (kcal/mol)	+1.79 (kcal/mol)
Sesamol	-5.13 (kcal/mol)	174.23 µM	-5.43 (kcal/mol)	-5.34 (kcal/mol)	-0.08 (kcal/mol)	+0.32 (kcal/mol)	+0.30 (kcal/mol)

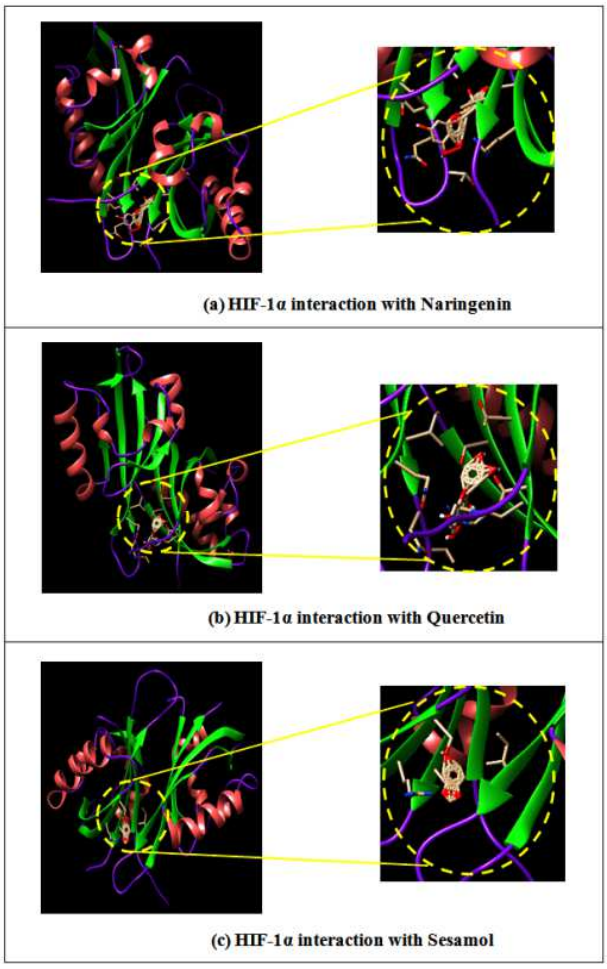


Fig. 5: Binding of HIF-1α with selected compounds

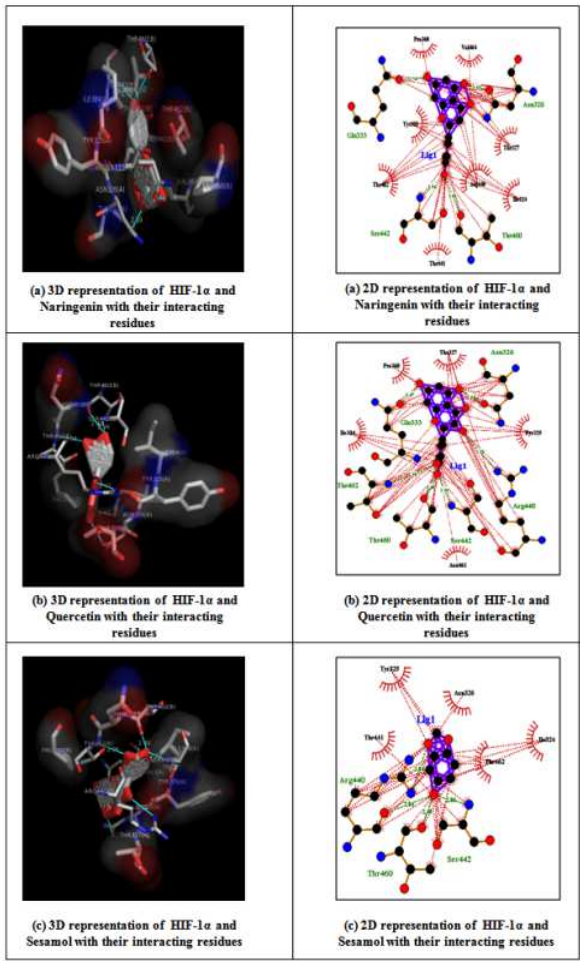


Fig. 6: 3D-and 2D-representation of HIF-1α and ligand interactio

Table 6: HIF-1α known inhibitory site and selected compounds interacting residues

Compounds	Interacting residues
Reported Active Site	ILE^{324}, TYR^{325}, ASN^{326}, THR^{327}, LYS^{328}, GLN^{333} and CYS^{334} of chain A and CYS^{358}, GLN^{359}, PRO^{360}, ARG^{362}, MET^{426}, ARG^{440}, THR^{441}, SER^{442}, THR^{460}, ASN^{461}, THR^{462}, ASN^{463}, VAL^{464} and LYS^{465} of chain B.
Naringenin	ILE^{324}, ASN^{326}, TYR^{325}, THR^{327} and GLN^{333} residues of chain A and PRO^{360}, ARG^{440}, THR^{441}, SER^{442}, THR^{460}, THR^{462} and VAL^{464} residues of chain B.
Quercetin	ILE^{324}, TYR^{325}, ASN^{326}, THR^{327} and GLN^{333} residues of chain A and PRO^{360}, ARG^{440}, SER^{442}, THR^{460}, ASN^{461} and THR^{462} residues of chain B.
Sesamol	ILE^{324}, TYR^{325} and ASN^{326} residues of chain A and ARG^{440}, THR^{441} SER^{442}, THR^{460} and THR^{462} residues of chain B.

DISCUSSION

Further, recent therapeutics advancement in hypoxia-mediated aberrations reveals the promising role of natural compounds as potent neuroprotective agents. By this *in the silico* investigation, we have successfully hunted 5 unique hits using BLAST [11] based on functional domain sequence and optimized the full-length genes of HIF-1α on the basis of families and domains identified from Interproscan results. These isoforms belong to Hypoxia-inducible factor, alpha subunit family (IPR021537), Hypoxia-inducible factor-1 alpha family (IPR001321), Myc-type, basic helix-loop-helix domain (IPR011598), PAS domain (IPR000014), PAS fold (IPR013767), PAS fold 3 (IPR013655), HIF-1 alpha, transactivation domain, C-terminal (IPR014887) and a repeat of PAC motif (IPR001610) and catalyse functions based on its activity to regulate the transcriptional activity in areas of vascularization and angiogenesis, energy metabolism, cell survival and tumour invasion. Further, the Phylogenetic study of HIF-1αrevealed that hypoxia-inducible factor 1α and HIF1A were in same cluster as they share the same homology and hypoxia-inducible factor 1αisoform 1 was in another cluster while Hypoxia-inducible factor 1-alpha isoform 3 and hypoxia-inducible factor 1αsubunit were differed from others [12]. ProtParam results showed that isoelectric point was 5.17 which indicates that protein was negatively charged thus it could be better for docking analysis. TheGRAVYindexis-0.573 for HIF-1α is indicative of hydrophilic and soluble protein. Template showed 99.07% sequence identity for HIF-1αprotein which is another important property for proper ligand interaction. 3D-structure of HIF-1αwas generated by using SWISS MODEL Server [13] and visualized using UCSF Chimera [14]. Z score for HIF-1αwas 0.81 respectively suggesting that input structure is within the range of scores typically found for native proteins of similar size. RAMPAGE displayed 99.5% of residues in the most favoured regions, 0.5% residues in additionally allowed and no residues in disallowed regions in HIF-1αProtein, showing that stereochemical quality of protein structure is good and which is important for proper docking. The result of Errat showed 95.694% accurate structure for HIF-1αprotein. Among the twenty-nine binding sites obtained from CastP Server for HIF-1α, site 29 was highly conserved within all the binding sites of HIF-1αprotein [15]. Further, active site prediction was performed since it is useful to

determine potential sites for ligand binding in molecular docking. Three compounds (*Naringenin, Quercetin* and *Sesamol*) obtained from different medicinal plants were selected for molecular docking study at *in silico* level. Lipinski Filter Analysis of all the compounds revealed that these compounds could act like a drug and have drug-like property as these compounds meet the criteria of Lipinski Rule of five [16]. Docking study revealed that all four compounds are interacting at the reported active and binding site [17, 18]. Inhibition Constant, Ki of *Naringenin, Quercetin* and *Sesamol* for HIF-1α was found to be 605.40 nM, 945.18 nM and 174.23 μM respectively suggesting that all the selected compounds are effective as HIF-1α inhibitors. Investigation of active and binding sites within HIF-1α protein and gives a better idea for a valuable drug target site and drug interaction with the highest affinity. In this result, the most effective compound was found to be *Naringenin* as showing minimum Inhibition Constant, Ki and highest negative free energy of binding with maximum interacting surface area [19-22].

CONCLUSION

In the light of the above analysis, we found that modulating the HIF-1α activity could be helpful for curing many disease progressions. Since, altered expression of this important transcription factor directs many abnormalities, including neuronal dysfunction and cancers. Further, *in silico* studies revealed that biomolecules might have a role in the inhibition of HIF-1α and in the prevention of hypoxia-mediated cellular dysfunction. All the biomolecules which have been selected for docking study are having drug-like property and may act as potential biomolecules for inhibiting or targeting the altered expression of HIF-1α. The *in silico* molecular docking study results revealed that all the biomolecules are having minimum binding energy and have good affinity toward the active pocket, thus, they may be supposed as good inhibitor of HIF-1α. Inhibition Constant, Ki of *Naringenin, Quercetin* and *Sesamol* for HIF-1α was found to be 605.40 nM, 945.18 nM and 174.23 μM respectively, advocating that all the selected compounds are effective as HIF-1α inhibitors. Additionally, investigation of active and binding sites within HIF-1α protein gives a better idea for a valuable drug target site and drug interaction with the highest affinity. Finally, in this result the most effective compound was found to be *Naringenin* as showing minimum inhibition constant, Ki and highest negative free energy of binding with the maximum interacting surface area. Thus, the role of natural compound *Naringenin* with HIF-1α provides a novel remedial approach among all three biomolecules based on docking studies and provide a potential curative biomarker for the treatment of patients suffering from hypoxic injuries.

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CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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Review

Impact of Insulin Degrading Enzyme and Neprilysin in Alzheimer's Disease Biology: Characterization of Putative Cognates for Therapeutic Applications

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Abstract. Alzheimer's disease (AD) is a neurodegenerative process primarily characterized by amyloid-β (Aβ) agglomeration, neuroinflammation, and cognitive dysfunction. The prominent cause for dementia is the deposition of Aβ plaques and tau-neurofibrillary tangles that hamper the neuronal organization and function. Aβ pathology further affects numerous signaling cascades that disturb the neuronal homeostasis. For instance, Aβ deposition is responsible for altered expression of insulin encoding genes that lead to insulin resistance, and thereby affecting insulin signaling pathway and glucose metabolism in the brain. As a result, the common pathology of insulin resistance between Type-2 diabetes mellitus and AD has led AD to be proposed as a form of diabetes and termed 'Type-3 diabetes'. Since accumulation of Aβ is the prominent cause of neuronal toxicity in AD, its clearance is the prime requisite for therapeutic prospects. This purpose is expertly fulfilled by the potential role of Aβ degrading enzymes such as insulin degrading enzyme (IDE) and Neprilysin (NEP). Therefore, their molecular study is important to uncover the proteolytic and regulatory mechanism of Aβ degradation. Herein, (i) *In silico* sequential and structural analysis of IDE and NEP has been performed to identify the molecular entities for proteolytic degradation of Aβ in the AD brain, (ii) to analyze their catalytic site to demonstrate the enzymatic action played by IDE and NEP, (iii) to identify their structural homologues that could behave as putative partners of IDE and NEP with similar catalytic action and (iv) to illustrate various IDE- and NEP-mediated therapeutic approaches and factors for clearing Aβ in AD.

Keywords: Alzheimer's disease, amyloid-β, insulin degrading enzyme, Neprilysin, therapeutics

INTRODUCTION

Alzheimer's disease (AD) is a neurological disorder that is characterized by neuronal death, which is caused by the abnormal burden of amyloid-β (Aβ) in the brain resulting in memory loss and cognitive decline [1–3]. The cognitive collapse in AD occurs due to neuronal dysfunction that is attributable to the extracellular Aβ

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aggregates and the intraneuronal aggregates of tau protein, which further forms Aβ plaques and neurofibrillary tangles, respectively [4–7]. Apart from Aβ deposition and neurofibrillary tangles, other characteristic abnormalities include dystrophic neurites, impaired energy metabolism, non-dynamic equilibrium between Aβ production and its clearance, chronic oxidative stress, mitochondrial dysfunction, DNA damage, elevated pro-apoptotic genes, and signaling pathways [8–11]. Currently researchers have reported that insulin and insulin-like growth factor resistance is another prominent cause provoking the progression of AD [12]. In addition, production of insulin in the brain, in addition to the pancreas, led to the proposal of another form of diabetes, termed 'Type-3 diabetes' for AD.

IS ALZHEIMER'S DISEASE A 'TYPE-3 DIABETES'? A MISNOMER OR A FACT

Insulin, insulin receptor (IR), and insulin–like growth factor-1 (IGF-1) play a prominent role in the brain via regulating brain metabolism, neuronal growth, and differentiation [13–15]. Any hindrance in the cross-talk between insulin and neuronal glucose metabolism may slow down ATP synthesis that culminates in neuronal apoptosis. This is in context with the dysfunction of energy metabolism linked with stress-induced fluctuations in the brain IR signaling cascade and the parallel death signals that caused apoptotic death [16, 17]. Mounting conclusive evidence uncovered the fact that diabetes and altered insulin signal may have a direct and indirect profound impact on progression of AD [18]. Diabetes mellitus is one of the most widespread metabolic disorders, primarily governed by interference in insulin signaling cascade, with prevalence in aged individuals. It is linked with gradually and highly progressive end-organ injury to the brain. Moreover, mild to moderate alterations of cognitive function have been clearly stated in both type 1 and type 2 diabetic (T1D and T2D, respectively) patients [19, 20]. The cardinal defect in T2D is insulin resistance, whose roots are relative insulin deficiency, and falls in the context of a cluster of metabolic and vascular risk factors, that is termed "metabolic syndrome." In this framework, a growing amount of evidence directly links insulin to cognitive decline and dementia in T2D [19, 20]. Furthermore, under dementia-type disorders, T2D has been increasingly linked with AD [21]. The connections between T2D and AD comprise high-cholesterol levels, aging-related processes, peripheral and central nervous system (CNS) insulin resistance, dysfunctional IR and IR signaling cascades, decreased glucose transport, and neurodegeneration. As a result of recurrent hyperinsulinemia/hypoglycemia in T2D, cognitive impairment promotes AD onset. Moreover, several epidemiology studies reported that T2D increased the risk of AD, and it is plausible that hyperinsulinemia plays a decisive role in AD progression [22–24].

Furthermore, de la Monte and colleagues presented compelling evidence for abnormalities in insulin/IR signaling in the brains of AD patients. These abnormalities greatly reduced CNS expression of genes encoding insulin, its receptors, and related signaling molecules in AD brain. Further, this situation promoted the researchers to consider sporadic AD as a "neuroendocrine disorder" that shared features with T1D and T2D, and they referred to it as "Type-3 diabetes", which closely resembles diabetes mellitus but still differs from it [12, 25, 26]. However, diabetic patients are highly prone (nearly 50 to 60%) to AD development due to insulin resistance in the brain, but non-diabetic patients are also developing AD. This reflects that in spite of the common features of insulin resistance in both T2D and AD (Fig. 1), they differ in their exact mechanism of occurrences. Still, it is debatable to consider AD as "Type-3 diabetes" and requires intense in-depth studies to unravel the mystery.

In AD brains, synaptic plasticity and neuronal survival are directly influenced by insulin resistance or indirectly by insulin degrading enzyme (IDE), which has been strongly advocated to be a key player in Aβ catabolism that catabolizes insulin and IGF-1, and also degrades Aβ [27, 28]. Moreover, insulin raises the extracellular concentration of Aβ via two independent mechanisms that include inhibition of extracellular degradation of Aβ by IDE or stimulation of Aβ secretion by the augmentation of its trafficking from the endoplasmic reticulum to the plasma membrane [29, 30]. Since, IRs in the brain do not desensitize itself, IDE creates a negative feedback loop to control insulin action. In fact, insulin exposure stimulates PI3-K/Akt-dependent upregulation of IDE, thereby preventing insulin signaling and promoting Aβ clearance in neurons. This evidence supports the existence of a therapeutic window allowing insulin treatment to adjust the levels of IDE in AD, without provoking direct competitive inhibition of Aβ breakdown. Insulin can be toxic for brain, if present in high levels, and creates both its positive and negative impact on brain metabolism, thus acting as a two-edged knife on the brain [31–33]. Apart from IDE, Aβ plaque formation is also downregulated by another degradative enzyme called Neprilysin (NEP) that is known to prevent AD progression.

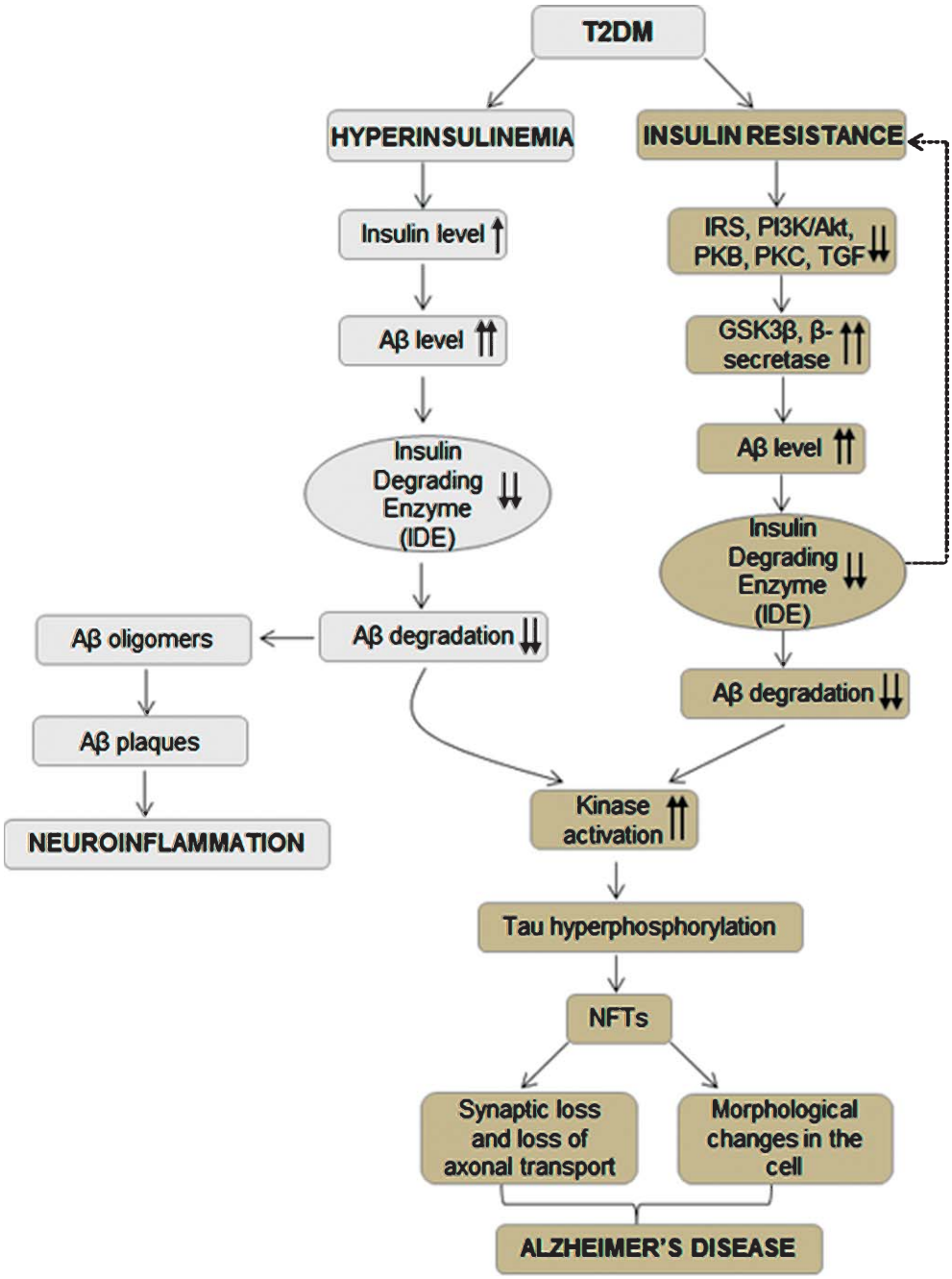

Fig. 1. Association of insulin degrading enzyme with the clinical symptoms of Alzheimer's disease as well as to the risk of type-2 diabetes mellitus.

NEPRILYSIN: NATURE'S AGENT FOR AD PREVENTION

NEP is a single-pass type II transmembrane protein, which has the single polypeptide chain of 750aa residues that is encoded by 2250 bp membrane metalloendopeptidase (MME) gene in humans [34, 35]. NEP has a molecular weight ranging from 85 to 110 kDa depending on its glycosylated isoforms [36, 37]. Among the human genome, the NEP gene is mainly located on chromosome 3 and exists in a single copy that covers >80 kb. Furthermore, it is composed of 24 exons and is found to be highly conserved across mammalian species [38]. It is a thermolysin-like zinc

metalloendopeptidase that requires one Zn^{2+} ion per subunit as a cofactor for its catalytic activity [39–41]. Moreover, a His-Glu-X-X-His (Zinc consensus sequence) is responsible for catalytic activity of NEP [42, 43]. NEP is also known as enkephalinase, kidney brush border neutral proteinase, endopeptidase, and neutral endopeptidase [44]. Previously, it has been identified as major atrial natriuretic peptide degrading enzyme expressed in kidney cell signifying its role in blood volume and pressure regulation [45]. However, its expression is much lower in several other tissues such as brain, where it is found on neuronal membranes (both pre- and post-synaptically), nigrostriatal bundle areas, hippocampal, and temporal cortex region, and is responsible for Aβ degradation in AD patients [46–48]. Moreover, in the case of the CNS, NEP is mainly expressed by neurons, activated astrocytes, and microglial cells, while in case of peripheral tissues, it is transiently expressed at the surface of certain haematopoietic cells [49–52].

NEP plays a crucial role in ceasing regulatory peptides (∼30 aa) that are actively involved in maintaining physiological homeostasis by altering the metabolic pathways of mammalian nervous, cardiovascular, immune, and inflammatory systems [53, 54]. Moreover, it possesses an ectoenzyme-like activity, which is involved in hydrolyzing extracellular oligopeptides at the N-terminal of amino acid residues that make it suitable for the degradation of the small hydrophobic $A\beta_{40\text{-}42}$ peptide [55]. The Aβ peptide degrading ability of NEP was first demonstrated by Howell et al. *in vitro* and later confirmed through *in vivo* experiments [56, 57]. NEP can digest both monomeric and toxic oligomeric forms of Aβ in the brain, and it has been demonstrated that irregular function of gene encoding NEP causes a two-fold increase in the endogenous toxic $A\beta_{40\text{-}42}$ levels (high plaque load) in different regions within the brain and thereby causing impaired synaptic plasticity and cognitive abnormalities that ultimately increase the risk of AD [58–60]. Further, it has also been observed that individuals with certain polymorphisms in the NEP gene have been associated with AD; for instance, a meta-analysis study has identified the association between NEP variants (rs989692 and rs3736187) and AD. These meta-analysis data also highlight that rs3736187 (A/G) polymorphisms might be a beneficial single nucleotide polymorphism (SNP), which is linked with a decreased risk in AD progression [61]. Interestingly, under hypoxic stress conditions such as cerebral ischemia, NEP activity gets affected due to altered AβPP processing by elevated expression of hypoxic products such as HNE [62]. This confirmed one possible mechanism by which NEP loses its activity and accumulation of Aβ in AD pathology and thus can correlate the role of NEP in Aβ clearance [63]. Apart from Aβ clearance, NEP also degrades several neuropeptides at the synapses such as enkephalins, substance P, tachykinins, neuropeptide-Y, and bradykinin. Besides the amyloid-degrading function of NEP, it is also involved in various functions within the brain which include memory and motor functions, synaptic plasticity, circadian rhythms, locomotion, sleep, anxiety, pain, blood-brain barrier (BBB) integrity, hyperalgesia, fatigue, water homeostasis, and neuroinflammation. Furthermore, its role has also been reported in the progression of a number of cancers, including renal, prostate, and lung cancer [64–66]. Since elucidation of the mechanistic role of any protein depends on its unique sequence and structure, its sequential and structural analyses have been done in order to gain additional molecular insights.

IN SILICO SEQUENTIAL AND STRUCTURAL ANALYSIS OF NEPRILYSIN

Primary structure analysis of NEP revealed it as a M13 family metallopeptidase (Source: InterPro) that preferentially cleaves polypeptides between the hydrophobic residues, especially Phe or Tyr. In order to understand the potential evolutionary relatives of NEP, BLAST tool has been used [67] and their sequence identity with NEP [*Homo sapiens*] is found to be (99%) NEP [*Macaca mulatta*], (99%) NEP [*Pongo abelii*], (94%) NEP [*Rattus norvegicus*], (94%) NEP [*Mus musculus*], and (91%) NEP [*Bos taurus*]. Moreover, multiple sequence alignments of these sequences (Fig. 2a) by ClustalW [68] revealed conserved zinc co-ordinating ligands at His584, His588, Glu646 [44], and active site aspartate residues at Asp591 and Asp651 [69] as well as 12 conserved cysteine residues that participate in the formation of six intra-subunit disulfide bonds (Cys57-Cys62, Cys80-Cys735, Cys88-Cys695, Cys143-Cys411, Cys234-Cys242, and Cys621-Cys747) [70]. Phylogenetic analysis of close relatives of NEP was performed by the neighbor-joining method [71] to construct a phylogenetic tree (Fig. 2b) that reflect evolutionary conservation between NEP [*Rattus norvegicus*] and NEP [*Macaca mulatta*], while NEP [*Homo sapiens*] as an out-group. The data obtained using ScanProsite [72] revealed one neutral zinc metallopeptidase (zinc-binding region) signature, which is important for its catalytic activity [73], 11 casein kinase II phosphorylation site, 11 protein kinase-C phosphorylation sites, one cAMP- and cGMP-dependent protein


10 20 30 40 50 60
gi|11625 (Homo sapiens) MGKSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|38678 (Macaca mulatta) MGRSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|19710 (Pongo abelii) MGKSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|69812 (Rattus norvegicus) MGRSESQMDI TDINAPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|31543 (Mus musculus) MGRSESQMDI TDINAPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|30079 (Bos Taurus) MGRSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
70 80 90 100 110 120
gi|11625 (Homo sapiens) DCIKSAARLI QNMDATTEPC TDFFKYACGG WLKRNVIPET SSRYGNFDIL RDELEVVLKD
gi|38678 (Macaca mulatta) DCIKSAARLI QNMDATAEPC ADFFKYACGG WLKRNVIPET SSRYGNFDIL RDELEVVLKD
gi|19710 (Pongo abelii) DCIKSAARLI QNMDATAEPC TDFFKYACGG WLKRNVIPET SSRYGNFDIL RDELEVVLKD
gi|69812 (Rattus norvegicus) DCIKSAARLI QNMDASAEPC TDFFKYACGG WLKRNVIPET SSRYSNFDIL RDELEVILKD
gi|31543 (Mus musculus) DCIKSAARLI QNMDASVEPC TDFFKYACGG WLKRNVIPET SSRYSNFDIL RDELEVILKD
gi|30079 (Bos Taurus) ECIKSAARLI QNMDATVEPC TDFFKYACGG WLKRNVIPET SSRYSNFDIL RDELEVVLKD
130 140 150 160 170 180
gi|11625 (Homo sapiens) VLQEPKTEDI VAVQKAKALY RSCINESAID SRGGEPLLKL LPDIYGWPVA TENWEQKYGA
gi|38678 (Macaca mulatta) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGEPLLKL LPDIYGWPVA TENWEQKYGA
gi|19710 (Pongo abelii) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGEPLLKL LPDVYGWPVA TENWEQKYGA
gi|69812 (Rattus norvegicus) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGQPLLTL LPDIYGWPVA SQNWEQTYGT
gi|31543 (Mus musculus) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGQPLLKL LPDIYGWPVA SDNWDQTYGT
gi|30079 (Bos Taurus) VLQEPKTEDI IAVQKAKMLY RSCINETAIE SRGGEPLLRL LPDIYDWPVA LENWEQKYGS
190 200 210 220 230 240
gi|11625 (Homo sapiens) SWTAEKAIAQ LNSKYGKKVL INLFVGTDDK NSVNHVIHID QPRLGLPSRD YYECTGIYKE
gi|38678 (Macaca mulatta) SWTAEKAIAQ LNSKYGKKVL INLFVGTDDK NSVNHVIHID QPRLGLPSRD YYECTGIYKE
gi|19710 (Pongo abelii) SWTAEKAIAQ LNSKYGKKVL INLFVGTDDK NSVNHVIHID QPRLGLPSRD YYECTGIYKE
gi|69812 (Rattus norvegicus) SWTAEKSIAQ LNSKYGKKVL INFFVGTDDK NSTQHIIHFD QPRLGLPSRD YYECTGIYKE
gi|31543 (Mus musculus) SWTAEKSIAQ LNSKYGKKVL INFFVGTDDK NSTQHIIHFD QPRLGLPSRD YYECTGIYKE
gi|30079 (Bos Taurus) SWTAEKSIAQ LNSKYGKKVI INFFVGTDDK NSTNHIIHID QPQLGLPSRD YYECTAAYKE
250 260 270 280 290 300
gi|11625 (Homo sapiens) ACTAYVDFMI SVARLIRQEE RLPIDENQLA LEMNKVMELE KEIANATAKP EDRNDPMLLY
gi|38678 (Macaca mulatta) ACTAYVDFMI SVARLIRQEE RLPIDENQLA LEMNKVMELE KEIANATAKP EDRNDPMLLY
gi|19710 (Pongo abelii) ACTAYVDFMI SVARLIRQEE RLPIDENQLA LEMNKVMELE KEIANATAKP EDRNDPMLLY
gi|69812 (Rattus norvegicus) ACTAYVDFMI SVARLIRQEQ RLPIDENQLS LEMNKVMELE KEIANATTKP EDRNDPMLLY
gi|31543 (Mus musculus) ACTAYVDFMI SVARLIRQEQ SLPIDENQLS LEMNKVMELE KEIANATTKP EDRNDPMLLY
gi|30079 (Bos Taurus) ACTAYVDFMI SVAKLIRQER GLPIDENQLS LEMNKVMDLE KEIANATTKS EDRNDPMLLY
310 320 330 340 350 360
gi|11625 (Homo sapiens) NKMTLAQIQN NFSLEINGKP FSWLNFTNEI MSTVNISITN EEDVVVYAPE YLTKLKPILT
gi|38678 (Macaca mulatta) NKMTLAQLQS NFSLEINGKP FSWLNFTNEI MSTVNISITN EEDVVVYAPE YLTKLKPILT
gi|19710 (Pongo abelii) KKMTLAQIQN NFSLEINGKP FSWLNFTNEI MSTVNISITN EEDVVVYAPE YLTKLKPILT
gi|69812 (Rattus norvegicus) NKMTLAKLQN NFSLEINGKP FSWSNFTNEI MSTVNINIQN EEEVVVYAPE YLTKLKPILT
gi|31543 (Mus musculus) NKMTLAKLQN NFSLEVNGKS FSWSNFTNEI MSTVNINIQN EEEVVVYAPE YLTKLKPILT
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gi|19710 (Pongo abelii) KYSARDLQNL MSWRFIMDLV SSLSRTYKES RNAFRKALYG TTSETATWRR CANYVNGNME
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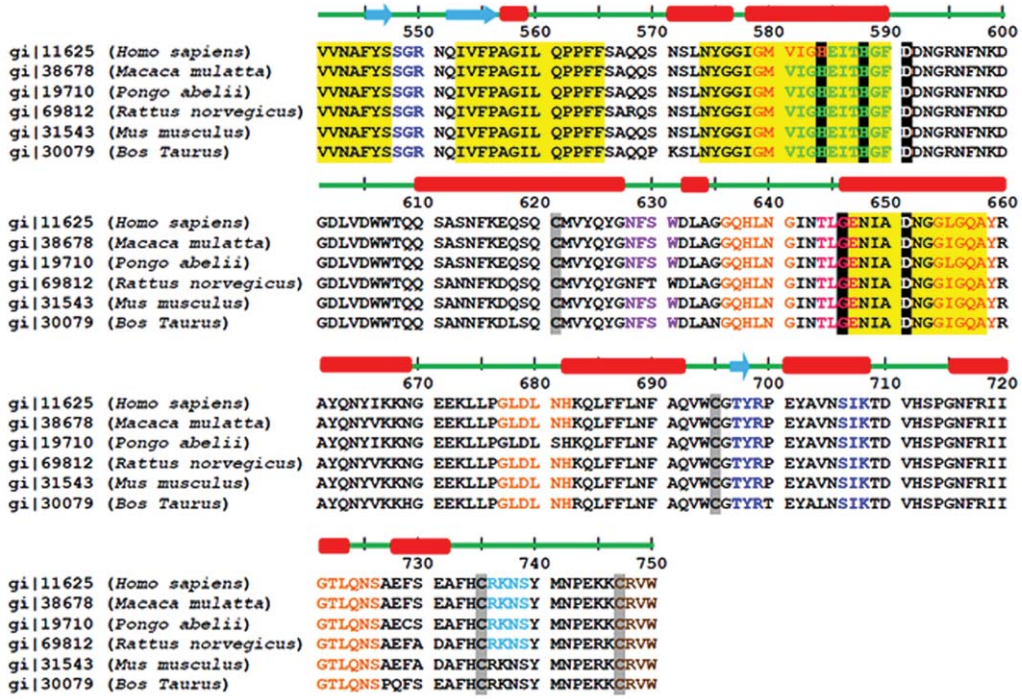
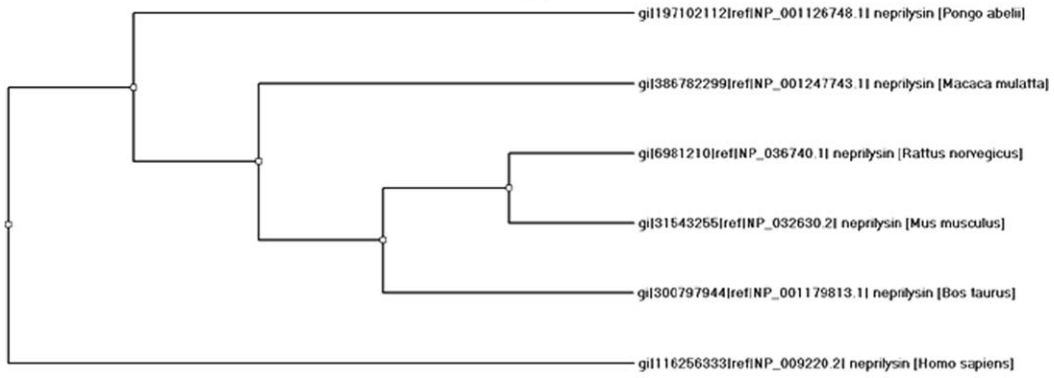


2(a)



2(b)

Fig. 2. a) Multiple sequence alignments of neprilysin (NEP) from different organisms with high sequence similarity. Motifs for NEP are highlighted in yellow color and conserved cysteine residues are highlighted in light grey color that is taking part in disulfide bond formation. Moreover, conserved zinc coordinating ligands His584, His588 and Glu646, and active site aspartate residues Asp591 and Asp651 are highlighted in black. Furthermore, secondary structural elements of NEP (*Homo sapiens*) obtained from DSSP are shown on the top of amino acid sequence as loop residues (green line), α-helices (red rectangles), and β-strands (blue arrows). Lastly, post translational modification sites are shown as colored amino acid residues with neutral zinc metallopeptidase region (light green), casein kinase II phosphorylation sites (pink), protein kinase-C phosphorylation sites (blue), N-myristoylation sites (orange), N-glycosylation sites (purple), amidation site (red), cAMP- and cGMP-dependent protein kinase phosphorylation site (light blue), and prenyl group binding site (brown). b) Phylogenetic relationship obtained by Neighbor-joining method using ClustalW. There is a single major cluster depicting evolutionarily highest conservation between NEP [*Rattus norvegicus*] and NEP [*Macaca mulatta*] with NEP [*Homo sapiens*] as out-group.

kinase phosphorylation site depicting their role in functional regulation of NEP via controlling their cellular locations, protein-protein interactions, and degradation by proteases. Moreover, there are 8 N-myristoylation sites and 6 N-glycosylation sites suggesting their role in membrane targeting and in imparting molecular stability to NEP, respectively [74, 75]. Lastly, one amidation site and one Prenyl group binding site (CAAX box) is also distributed well on the primary structure of NEP.

Table 1
List of sequence motifs present on Neprilysin identified by FingerPRINTScan

S. No.	Motif Name	Signature sequence	Id Score	Pf Score	p value	Sequence position
1	NEPRILYSIN (Neprilysin metalloprotease (M13) family signature)	WISGAAVVNAFYS	55.44	388	2.95E-05	535 to 548
		IVFPAGILQPPFF	82.23	567	1.81E-09	553 to 566
		NYGGIGMVIGHEITHGF	77.28	790	5.52E-12	574 to 591
		ENIADNGGLGQA	79.89	466	3.34E-07	647 to 659
2	CUATPASEI (Copper-transporting ATPase 1 signature)	TVNISITNEEDVVVYAPE	20	258	2.46E-03	333 to 351
		GAAVVNAFYSSGRNQIVFPAGILQ	17.5	180	6.27E-02	538 to 562
3	NRPEPTIDEY5R (Neuropeptide Y5 receptor signature)	EDEYFENIIQNLKFSQSKQL	34	223	5.20E-02	504 to 524
		DLVDWWTQQSASNFKEQSQCMVYQ	21.67	264	4.15E-03	602 to 626
4	RECA (RecA protein signature)	LKDVLQEPKTEDIVAVQKAKALYRSCINES	21.86	183	3.16E-02	118 to 148
		LGQAYRAYQNYIKKNGEE	25.28	191	1.17E-02	655 to 673
5	MTABOTROPICR (Metabotropic glutamate receptor signature)	FENIIQNLKFSQS	26.32	158	3.66E-02	508 to 521
		GGIGMVIGHEITHGFD	27.63	167	1.48E-02	576 to 592
6	DELTATUBULIN (Delta-tubulin signature)	DFMISVARLIRQEERLPIDEN	23.81	225	1.14E-02	247 to 268
		VGRLYVEAAFAGESKHVVED	22.5	155	8.11E-02	423 to 443
7	TNFC (GTP-binding elongation factor signature)	LVLLLTIIAVTMIALYATYDDG	27.27	200	3.24E-02	34 to 56
		EQKYGASWTAEKAIAQLNSKYGK	21.74	190	3.49E-02	175 to 198
8	TSHRECEPTOR (Thyrotropin receptor precursor signature)	SDCIKSAARLIQNMDATTE	20.18	190	8.36E-02	60 to 79
		GTLQNSAEFSEAFHCRKNSYMNPEKK	17.31	232	1.78E-02	721 to 747
9	SPLICEFRBRR1 (Pre-mRNA-splicing factor BRR1 signature)	AQLNSKYGKKVLINL	21.21	218	4.87E-02	189 to 204
		TKLKPILTKYSARDL	29.7	180	3.67E-02	353 to 368
10	AQUAPORIN10M (Mammalian aquaporin-10 signature)	RCANYVNGNME	36.36	230	3.30E-02	410 to 421
		SGAAVVNAFY	50	183	5.46E-02	537 to 547

Further, sequence motif analysis by FingerPRINTScan [76], identified motifs in 10 different families (Table 1) that belong to certain proteases, receptors, and factors signifying their involvement in cellular metabolic biological processes, such as modulation of peptides and regulation of signal transduction.

Furthermore, structural analysis of NEP (PDBID: 1R1H) revealed the presence of 43 α-helices (57%) and 15 β-strands (7%), which suggest that NEP is an α-rich protein (Source: DSSP) [77]. Structural classification of proteins or SCOP [78] analysis of NEP sequence suggested that it belonged to class of α and β proteins, with a Zinc-like fold and neutral endopeptidase family, and are classified as hydrolases (EC3.4.24.11). It is comprised of three domains viz. cytoplasmic (2–28), transmembrane (29–51), and extracellular (52–750). The structural topology of NEP is illustrated in Fig. 3, which is important to understand the protein unfolding mechanisms during its catalytic activity. NEP carries His583, His587, and Glu646 as coordinating ligands for Zn^{2+} ion [44, 79] and Asp590, Asp650 is assumed to form an active site triad along with coordinating histidine residues for its peptidase activity [69]. Moreover, homologous structures of NEP in the protein data bank have been identified by DALI server [80] (Table 2) that displayed its close structural similarity with endothelin-converting enzyme 1 (PDBID-3DWB) with Zscore: 49.6 and RMSD: 1.7. The obtained putative partners belong to proteases and co-proteases indicating their role in peptide cleavage or degradation.

INSULIN DEGRADING ENZYME: A THERAPEUTIC AGENT FOR Aβ DEGRADATION

IDE is an intracellular protease of 1019aa synthesized from a large reading frame of 3416 bp [81]. It is a single polypeptide chain of 110 kDa that naturally exists as a homodimer and requires one Zn^{2+} ion per subunit as a cofactor for its proteolytic activity [82, 83]. It is a thiol dependent, zinc metalloprotease that ubiquitously highly expressed in the brain, testes, liver, and muscles [84, 85]. IDE is principally located in cytosol, as well as on cell-surface [86, 87], although it has also been spotted within sub cellular organelles like mitochondria, endosomes, and peroxisomes, where its degradative role has been seen against oxidized proteins [88–90]. It is commonly known as insulysin or insulinase due to its well-known action against insulin and is located on chromosome 10q24, which is genetically linked with late-onset of AD genes [91, 92]. IDE possesses His-X-X-Glu-His (a zinc binding motif) at its active site that is common in a number of other eukaryotic and prokaryotic zinc peptidases [93]. Moreover, IDE has the potential to recognize the secondary and tertiary structures present in its substrates that enable it to recognize and cleave numerous small peptides, which include biologically active hormones and disease-related peptides, such as soluble amyloidogenic peptides, glucagon, calcitonin, amylin, atrial natriuretic peptide, insulin, and IGF-1

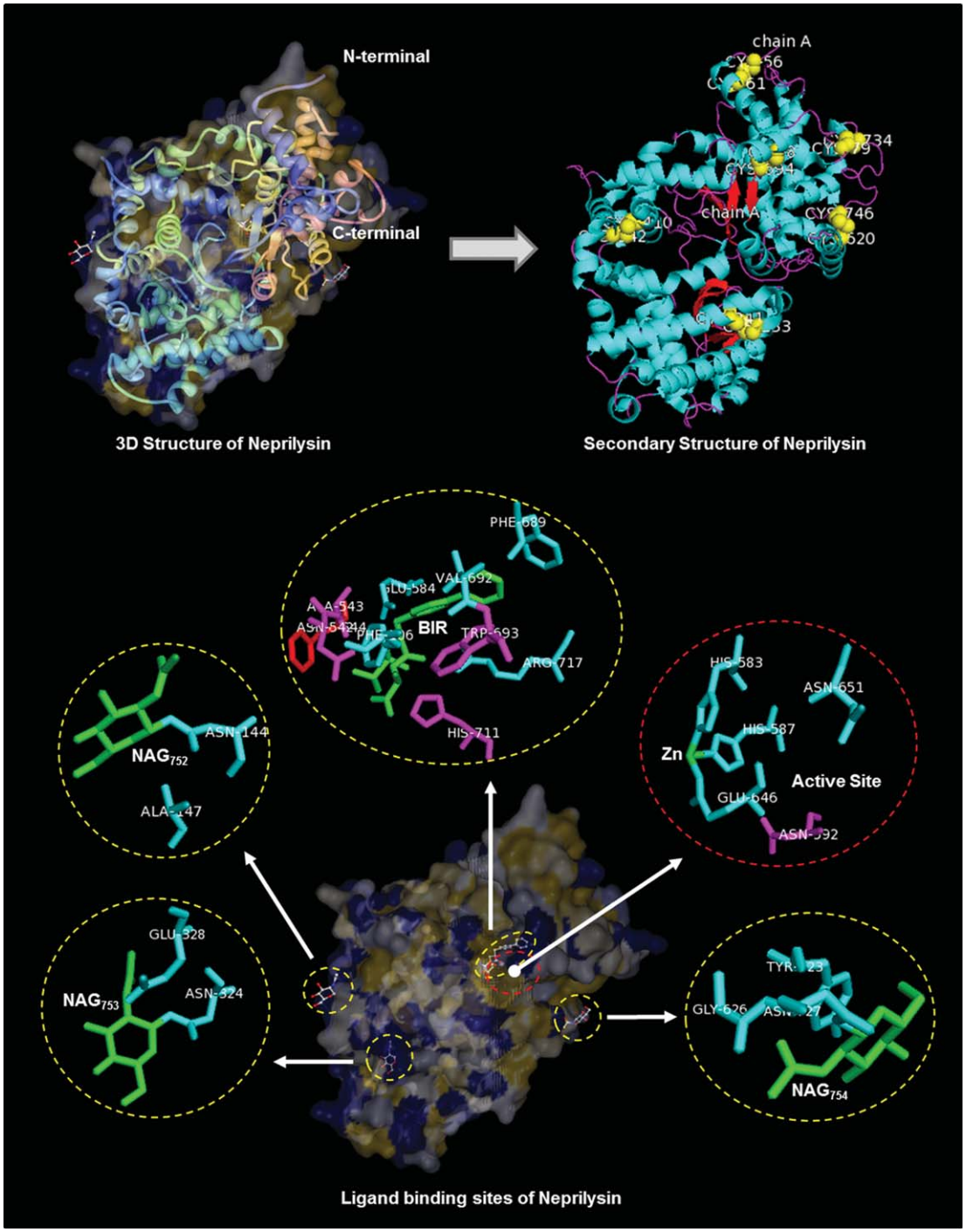


Fig. 3. 3D architecture of Neprilysin (PDB ID: 1R1H). Secondary structure of protein has 43 helices (blue), 15 strands (red), and loops (pink) where disulfide bridges are shown as yellow spheres (Cys56-Cys61, Cys79-Cys734, Cys87-Cys694, Cys142-Cys410, Cys233-Cys241, and Cys620-Cys746). Ligand binding sites of Neprilysin: Ligands are shown in green while ligand binding residues are shown in blue (helix forming residues), red (strand forming residues), and pink (loop residues). NAG (N-acetyl-D-glucosamine)-NAG_{752} binding residues-Asn144, Ala147; NAG_{753} binding residues-Asn324, Glu328; NAG_{754} binding residues-Tyr623, Gly626, Asn627; BIR (N-[3-[(1-aminoethyl)(Hydroxy)Phosphoryl]-2-(1,1'-biphenyl-4-ylmethyl)Propanoyl]Alanine) binding residues-Phe106, Asn542, Ala543, Phe544, Glu584, Phe689, Val692, Trp693, His711, Arg717; Zinc metal binding residues-His583, His587, Glu646, and active site residues-Asp591, Asp650.

Table 2
Ten potential putative partners of Neprilysin identified by DALI server

Name of Protein	PDB Code	Number of residues		Z-Score	RMSD	Sequence Identity (%)
		Total	Super-imposed			
Neprilysin	1R1H	696	696	69	0	100
Endothelin-Converting Enzyme 1	3DWB	661	658	49.6	1.7	41
Endopeptidase, Peptidase Family M13	3ZUK	658	641	44	2.2	31
Neutral Endopeptidase	4IUW	630	618	42	2.6	23
Peptidase, M48 Family	3C37	222	143	6.9	4	18
Endoplasmic Reticulum Aminopeptidase 2	3SE6	870	195	6.7	5.2	12
Tricorn Protease Interacting Factor F3	1Z1W	780	184	6.6	4.9	13
Glutamyl Aminopeptidase	4KXB	875	205	6.5	5.1	9
Thermolysin	3EIM	316	150	6.4	3.4	16
Secreted Metalloprotease MCP02	3NGX	299	144	6.2	3.4	18

and 2 at physiological pH [87, 94–96]. Recently, IDE has been identified as the protease responsible for the conversion of β-endorphin to γ-endorphin, signifying its role in signal transduction in the brain [97, 98]. Although it identifies amyloidogenic peptides, it can cleave them only in their monomeric soluble form and not in their aggregated form [43, 99]. It also digests cytoplasmic and monomeric Aβ peptides [100]. However, these peptides contain different proportions of α-helix and β-sheet structure in solution, but upon enzyme action or self-aggregation, they assume the β-sheet conformation. Multiple studies indicate that IDE provides a surface for its substrates to assume a β-sheet, where the residues interacting with IDE are same that are responsible for oligomerization and fibrillization of amyloidogenic peptides [101]. Thus, IDE acts as an amyloid-scavenging enzyme, hampering the formation of amyloid plaques, and nullifies their toxic effects [102]. Further studies identified the consensus sequence responsible for IDE-substrate interaction, i.e., '*hnhhhpsh*' where h represents hydrophobic, n (small, neutral), p (polar), and s (polar and/or small) amino acid residues [96, 103, 104].

Typical AD pathology, such as formation of amyloid-plaques, is found to be exacerbated by insulin dysregulation in the brain where insulin resistance and low insulin levels have been seen. IRs occupy neuronal synapses and astrocytes in the memory-processing brain regions such as cerebral cortex, hippocampus, olfactory bulb, cerebellum, and hypothalamus [105]. At the molecular level, when insulin binds with IR, it triggers certain signaling cascade that is mainly associated with the formation of long-term memory and learning, which comprised of intracellular signaling molecules; for instance, Grb-r/SOS, shc, Ras/Raf, and MEK/MAP kinases. Another signaling cascade, which includes IRS-1, PI3K/Akt/GSK-3, protein kinase-B/C, and non-receptor tyrosine kinase-pp60c-src molecules are also coupled with memory processing [106–108] and further activate other factors like IGFs and transforming growth factors [109]. Any dysregulation in the level of these factors in the brain imparts insulin resistance to the IRs [110, 111]. In addition, when insulin levels reach to significantly high level, Aβ, a major culprit neurotoxin in AD, starts to accumulate in senile plaques, resulting in neuroinflammation. Further, it is evidenced by the fact that exaggerated plasma insulin levels lead to an increased burden of Aβ peptide in the cerebrospinal fluid, resulting in memory loss; therefore, AD is also characterized as "neuroendocrine disorder" associated with insulin signaling [8, 112]. Insulin plays a crucial role in controlling neurotransmitter release at the synapses and triggers signaling cascades associated with learning/long-term memory, energy metabolism, glucose utilization, and neuronal survival [113]. It not only regulates the blood-sugar level but also acts as a growth factor on all cells, including neurons in CNS; therefore, any disturbances in insulin signaling might hamper cellular repair mechanisms, cell growth/differentiation, and provoke numerous degenerative processes.

IN SILICO SEQUENTIAL AND STRUCTURAL ANALYSIS OF INSULIN DEGRADING ENZYME

Primary structure analysis of IDE revealed it as a metalloenzyme and M16 family metallopeptidase such as s-ribosyl homocysteinase and mitochondrial processing peptidase respectively (Source: InterPro). Potential evolutionary relatives of IDE [*Homo sapiens*] were obtained by BLAST [67] that revealed its sequence identity as follows: (99%) IDE [*Macaca mulatta*], (98%) IDE precursor [*Bos taurus*], (98%) IDE [*Bos mutus*], (95%) IDE [*Mus musculus*]. Further,

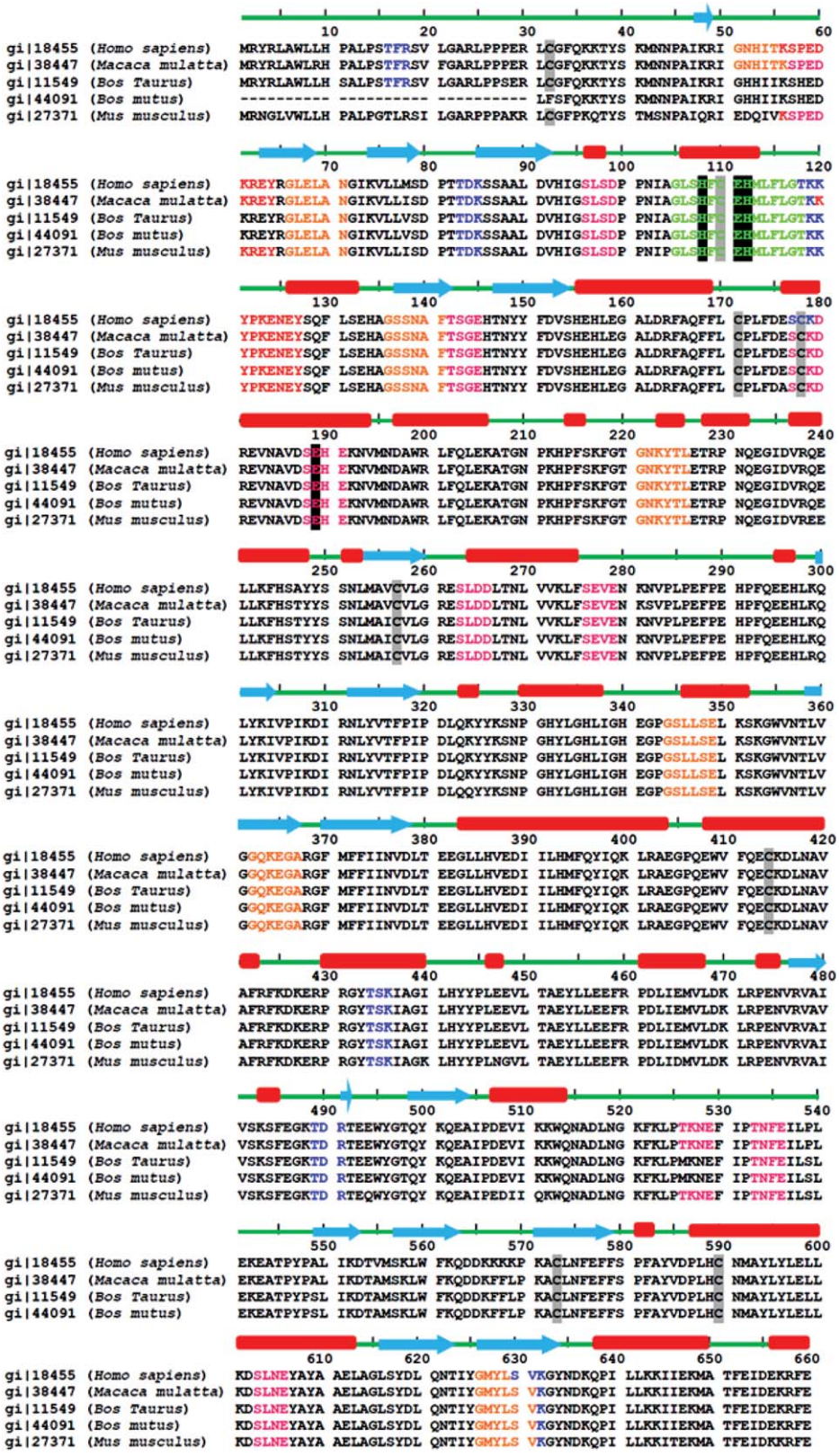
multiple sequence alignments of these sequences (Fig. 4a) by ClustalW [68] revealed 11 conserved cysteine residues signifying their role in regulation of IDE via oxidative or nitrosative processes; Zn^{2+} ion coordinating ligands at His108, His112, and Glu189 [114] and a conserved glutamate residue (Glu111) at the active site [83]. Phylogenetic analysis of a close relative of IDE was performed by the neighbor-joining method [71] to obtain a phylogenetic tree (Fig. 4b) that indicated evolutionary conservation between IDE precursor [*Bos taurus*] and IDE partial [*Bos mutus*], while IDE [*Homo sapiens*] as an out-group. Further, analysis using ScanProsite [72] identified one insulinase family (zinc-binding region) signature, which is important for its catalytic activity [115], 10 protein kinase-C phosphorylation sites, 5 tyrosine kinase phosphorylation sites, 16 casein kinase II phosphorylation sites, one cAMP- and cGMP-dependent protein kinase phosphorylation site depicting their role in functional regulation of IDE via controlling their cellular locations, protein-protein interactions, and degradation by proteases. Moreover, there are seven N-myristoylation sites and three N-glycosylation sites suggesting their role in membrane targeting and in imparting molecular stability to IDE, respectively [74, 75]. Finally, one microbodies C-terminal targeting the signal sites is also distributed well on the primary structure of IDE.

Further, sequence motifs analysis by FingerPRINTScan revealed motifs in nine different families of proteins (Table 3) that belong to certain transcription factors and nuclear receptors depicting their significant role in signaling processes.

Structural analysis of IDE (PDB ID: 4IFH) revealed the presence of 44 α-helices (41%) and 32 β-strands (19%), which suggest that IDE is an α-rich protein (Source: DSSP) [77] and belongs to the peptidase family M16 (EC 3.4.24.56) and has two isoforms: membrane associated and cytoplasmic. Further, its quaternary structure comprised of homodimers where it requires one Zn^{2+} ion per subunit for its peptidase activity. The structural topology of IDE is illustrated in Fig. 5, which is important for the understanding of protein unfolding mechanisms during its catalytic activity. IDE carries His108, His112, and Glu189 as coordinating ligands for Zn^{2+} ion [114] and Glu111 at active site contributing to its catalytic activity [83]. Moreover, homologous structures of IDE in the protein data bank have been identified by DALI server [80] (Table 4) that displayed its close structural similarity with Protease III (PDB ID: 1Q2L) with Z score: 30.8 and RMSD: 7.1. The obtained putative partners belong to different peptidases suggesting their role in peptide cleavage or degradation.

INSULIN DEGRADING ENZYME AND NEPRILYSIN MEDIATED Aβ CLEARANCE

Aβ accumulation is one of the characteristic features of AD that contributes to plaque formation and provokes the formation of toxic oligomeric Aβ complexes in the brain. The main component of plaques is the hydrophobic Aβ which is an approximately 4.2 kDa peptide formed mainly by proteolytic cleavage of the amyloid-β protein precursor (AβPP) by the action of proteolytic enzymes. The proteolytic cleavage is accompanied by two pathways, non-amyloidogenic (α-secretase) and amyloidogenic (β- and γ-secretase). The α-secretase cleavage site is mainly situated at position 16 within the Aβ sequence precluding the generation of Aβ, while amyloidogenic AβPP processing the main pathway of AβPP cleavage in cells that takes place at the plasma membrane. β- and γ-secretases produce various biologically active metabolites, including the Aβ and the AβPP intracellular domain (AICD) by virtue of proteolytic cleavage of AβPP [116]. Furthermore, the γ-secretase complex consists of four major proteins, including PS1 or PS2, Aph (anterior pharynx defective) 1a, presenilin enhancer2 (PEN2), and nicastrin. It cleaves its substrates within the membrane, a process often involved in important signaling cascades and termed regulated intramembrane proteolysis [35]. Since the catalytic subunit of γ-secretase is either PS1 or PS2, any mutations in AβPP or the multi-subunit protease complex γ-secretase components are one of the reasons for accumulation of Aβ that may lead to early-onset of familial AD [117]. Besides the *de novo* synthesis of Aβ caused by amyloidogenic processing of AβPP, Aβ levels are also highly dependent on Aβ degradation. Proteolytic cleavage by β- and γ-secretases following amyloidogenic cascade makes Aβ neurotoxic, while proteolytic cleavage by α-secretase makes soluble Aβ, which is neuroprotective in nature. There are two major proteases (IDE and NEP) involved in Aβ clearance that act on different targets responsible for Aβ deposition in the brain (Fig. 6). It has been observed that overexpression of IDE resulted in degradation of AICD and insulin to significantly reduced Aβ burden in AD patients [81, 118]. Furthermore, AICD binds to the NEP promoter and leads to transcriptional activation of NEP by competitive replacement with histone deacetylases. This transcriptional activation of NEP raises its expression in the brain and leads to Aβ clearance [119]. Recently, it has been identified that long isoform of IDE (IDE-Met1) is also involved in Aβ clearance following mitochondrial biogenesis path-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gi|38447 (Macaca mulatta) MRYRLAWLRH PALPSTFRSV FGARLPPPER LCGFQKKTYS KMNNPAIKRI GNHITKSPED
gi|11549 (Bos Taurus) MRYRLAWLLH SALPSTFRSV LGARLPPSER LCGFQKKTYS KMNNPAIKRI GHHIIKSHED
gi|44091 (Bos mutus) ---------- ---------- ---------- LFSFQKKTYS KMNNPAIKRI GHHIIKSHED
gi|27371 (Mus musculus) MRNGLVWLLH PALPGTLRSI LGARPPPAKR LCGFPKQTYS TMSNPAIQRI EDQIVKSPED
70 80 90 100 110 120
gi|18455 (Homo sapiens) KREYRGLELA NGIKVLLMSD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|38447 (Macaca mulatta) KREYRGLELA NGIKVLLISD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|11549 (Bos Taurus) KREYRGLELA NGIKVLLVSD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|44091 (Bos mutus) KREYRGLELA NGIKVLLVSD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|27371 (Mus musculus) KREYRGLELA NGIKVLLISD PTTDKSSAAL DVHIGSLSDP PNIPGLSHFL EHMLFLGTKK
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gi|38447 (Macaca mulatta) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|11549 (Bos Taurus) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|44091 (Bos mutus) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|27371 (Mus musculus) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDASCKD
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gi|27371 (Mus musculus) REVNAVDSEH EKNVMNDAWR LFQLEKATGN PKHPFSKFGT GNKYTLETRP NQEGIDVREE
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gi|44091 (Bos mutus) LLKFHSTYYS SNLMAICVLG RESLDDLTNL VVKLFSEVEN KNVPLPEFPE HPFQEEHLKQ
gi|27371 (Mus musculus) LLKFHSTYYS SNLMAICVLG RESLDDLTNL VVKLFSEVEN KNVPLPEFPE HPFQEEHLRQ
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gi|44091 (Bos mutus) LYKIVPIKDI RNLYVTFPIP DLQKYYKSNP GHYLGHLIGH EGPGSLLSEL KSKGWVNTLV
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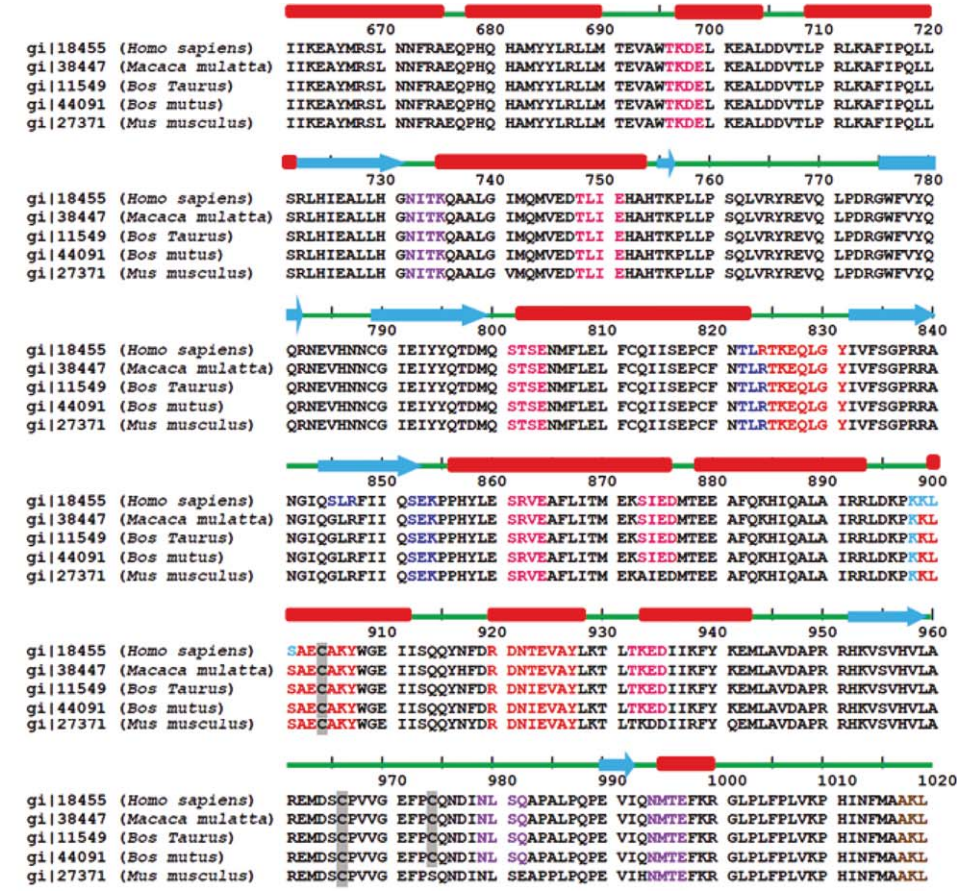
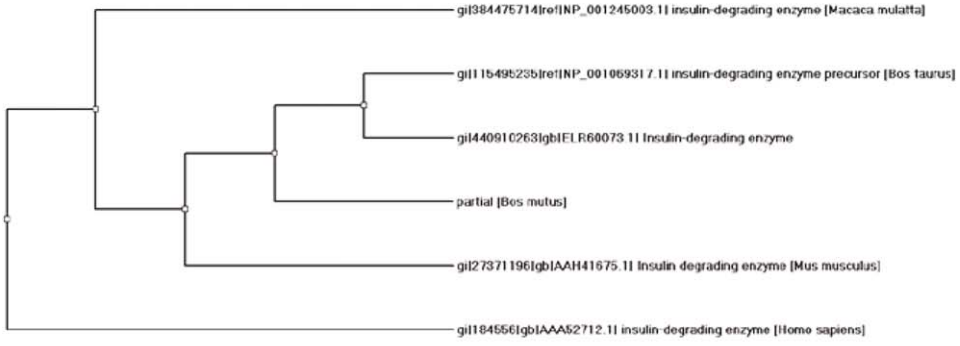
gi|44091 (Bos mutus) AFRFKDKERP RGYTSKIAGI LHYYPLEEVL TAEYLLEEFR PDLIEMVLDK LRPENVRVAI
gi|27371 (Mus musculus) AFRFKDKERP RGYTSKIAGK LHYYPLNGVL TAEYLLEEFR PDLIDMVLDK LRPENVRVAI
490 500 510 520 530 540
gi|18455 (Homo sapiens) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPTKNEF IPTNFEILPL
gi|38447 (Macaca mulatta) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPTKNEF IPTNFEILPL
gi|11549 (Bos Taurus) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPMKNEF IPTNFEILSL
gi|44091 (Bos mutus) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPMKNEF IPTNFEILSL
gi|27371 (Mus musculus) VSKSFEGKTD RTEQWYGTQY KQEAIPEDII QKWQNADLNG KFKLPTKNEF IPTNFEILSL
550 560 570 580 590 600
gi|18455 (Homo sapiens) EKEATPYPAL IKDTVMSKLW FKQDDKKKKP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
gi|38447 (Macaca mulatta) EKEATPYPAL IKDTAMSKLW FKQDDKFFLP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
gi|11549 (Bos Taurus) EKEATPYPSL IKDTAMSKLW FKQDDKFFLP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
gi|44091 (Bos mutus) EKEATPYPSL IKDTAMSKLW FKQDDKFFLP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
610 620 630 640 650 660
gi|18455 (Homo sapiens) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|38447 (Macaca mulatta) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|11549 (Bos Taurus) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|44091 (Bos mutus) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|27371 (Mus musculus) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKITEKMA TFEIDKKRFE



4(a)



4(b)

Fig. 4. a) Multiple sequence alignment of insulin degrading enzyme (IDE) from different organisms with high sequence similarity. Conserved cysteine residues are highlighted in gray while Zn^{2+} ion coordinating ligands His108, His112, and Glu189, and a conserved active site glutamate (Glu111) residue is highlighted in black. Further, secondary structural elements of IDE (*Homo sapiens*) obtained from DSSP are shown on the top of amino acid sequence with loop residues (green line), α-helices (red rectangles), and β-strands (blue arrows). Moreover, potential sites are shown as colored amino acid residues as follows insulinase family (zinc binding region) signature (light green), casein kinase II phosphorylation sites (pink), protein kinase-C phosphorylation sites (blue), N-myristoylation sites (orange), N-glycosylation sites (purple), tyrosine kinase phosphorylation sites (red), cAMP- and cGMP-dependent protein kinase phosphorylation site (light blue), and microbodies C-terminal targeting signal (brown). b) Phylogenetic relationship obtained by Neighbor-joining method using ClustalW. There is a single major cluster depicting evolutionarily highest conservation between IDE precursor [*Bos taurus*] and IDE partial [*Bos mutus*] with IDE [*Homo sapiens*] as an out-group.

Table 3
List of sequence motifs present on IDE identified by FingerPRINTScan

S.No.	Motif Name	Signature sequence	Id Score	Pf Score	*p* value	Sequence position
1	LYMPHOTACTNR (Lymphotactin	LGRESLDDLTNLVV	39.29	295	4.36E-03	259 to 273
	receptor signature)	FRFKDKERPRG	40.91	215	6.09E-02	422 to 433
2	F138DOMAIN (FAM138 N-	GIKVLLMSDPTTDK	30.95	192	3.28E-03	72 to 86
	terminal domain signature)	LSDPPNIAGLSHFCEH	26.39	127	8.16E-02	97 to 113
3	LVIRUSORF2 (Luteovirus ORF2	HAGSSNAFTSGEHTNYYFDVSH	25.97	221	4.22E-02	134 to 156
	putative replicase 1 signature)	LIEMVLDKLRPENVRVAIV	15.79	178	2.03E-02	463 to 482
4	SIGMA70FCT (Major sigma-	NITKQAALGIMQMV	18.57	189	2.80E-02	732 to 746
	70 factor signature)	QKHIQALAIRRL	30.48	167	3.12E-02	883 to 894
5	CYTOCHROMEF (Cytochrome	LYKIVPIKDIRNLYVTFPIPD	23.4	174	9.01E-02	301 to 322
	F signature)	IEMVLDKLRPENVRVAIV	34.3	202	3.57E-02	464 to 482
6	NUCLEARECPTR (Orphan	EEFRPDLIEMVLDKLRPE	20.71	177	3.30E-02	457 to 475
	nuclear receptor (4A nuclear	CAKYWGEIISQQYNFD	29.55	218	9.99E-02	904 to 920
	receptor) family signature)					
7	FLGFLGJ (Flagellar	SPFAYVDPLHCNMAYLYLELLKDS	16.67	210	6.78E-02	580 to 604
	protein FlgJ signature)	KSIEDMTEEAFQKHIQAL	22.22	220	5.05E-02	872 to 890
8	COUPTNFACTOR (COUP transcription	NFEILPLEKEATPYPA	32.5	179	9.54E-02	534 to 550
	factor (2F nuclear receptor)	PRLKAFIPQLLSRL	26.19	180	4.91E-02	710 to 724
	family signature)					
9	NISCPROTEIN (Nisin biosynthesis	KDKERPRGYTSKIAGIL	29.41	240	9.03E-02	425 to 442
	protein NisC signature)	LIKDTVMSKLWFKQDDKKKKPKAC	25	230	7.99E-02	550 to 574

Table 4
Ten potential putative partners of IDE identified by DALI server

Name of Protein	PDB Code	Number of residues		Z-Score	RMSD	Sequence Identity (%)
		Total	Superimposed			
Insulin-Degrading Enzyme	4IFH	956	955	60.2	0	100
Protease III	1Q2L	937	684	30.8	7.1	28
Zinc Metalloprotease	2FGE	979	801	20.1	4.2	12
Falcilysin	3S5K	1053	808	17.8	4.3	13
Zinc Peptidase	3AMI	422	406	16.4	3.4	17
Ubiquinol-Cytochrome-C Reductase Complex Core Pro	2A06	442	412	15.6	3.5	17
NADH-Quinone Oxidoreductase Subunit 1	2YBB	442	412	15.5	3.5	17
Mitochondrial Processing Peptidase Alpha Subunit	1HR7	440	413	15.5	3.5	16
Peptidase M16 Inactive Domain Family Protein	3GWB	412	395	15.5	3.6	14
Cytochrome BC1 Complex	1BGY	446	403	14.7	3.5	15

way. IDE-Met1 links the mitochondrial biogenesis pathway with mitAβ levels and organelle functionality. Activation of PGC1-α by the effect of mitochondrial biogenesis stimuli promotes NRF-1 expression, which makes long (IDE-Met1) and the short (IDE-Met42) IDE isoforms. Furthermore, the long IDE isoform is involved in mitochondrial Aβ clearance without any toxic effect [120]. Another important candidate for Aβ clearance is ApoE, which acts within microglia and in the extracellular space to affect the clearance of Aβ through promoting its proteolysis by IDE and NEP. The endolytic degradation of Aβ peptides within microglia is facilitated by NEP, while extracellular Aβ is degraded by IDE. Since the ability of ApoE to promote Aβ degradation is dependent upon the ApoE isoform and its lipidation status, lipidated ApoE is formed by transfer of lipids to ApoE, which is accomplished mainly by ABCA1 and LXR activation in the nucleus. It has also been reported that one isoform of ApoE, ApoE4, is associated with higher risk of AD, while the ApoE2 and ApoE3 isoforms are associated with lower risk of AD compared with ApoE4 [121]. Furthermore, the receptor for the advanced glycation end products (RAGE) act as a transporter of Aβ across the BBB into the CNS where it deposits Aβ, while the low-density lipoprotein receptor-related protein (LRP) mediates extra burden of Aβ outside the brain. Both IDE and NEP are also involved in RAGE-mediated Aβ degradation inside the brain, while ApoE, ApoJ, and α_2-macroglobulin are involved in Aβ transportation outside the brain in liver for degradation through LRP complex [122]. Since RAGE assists in Aβ accumulation inside the neuron, IDE and NEP bind to RAGE and blocks their functions in order to maintain memory cognition and neuronal survival [123].

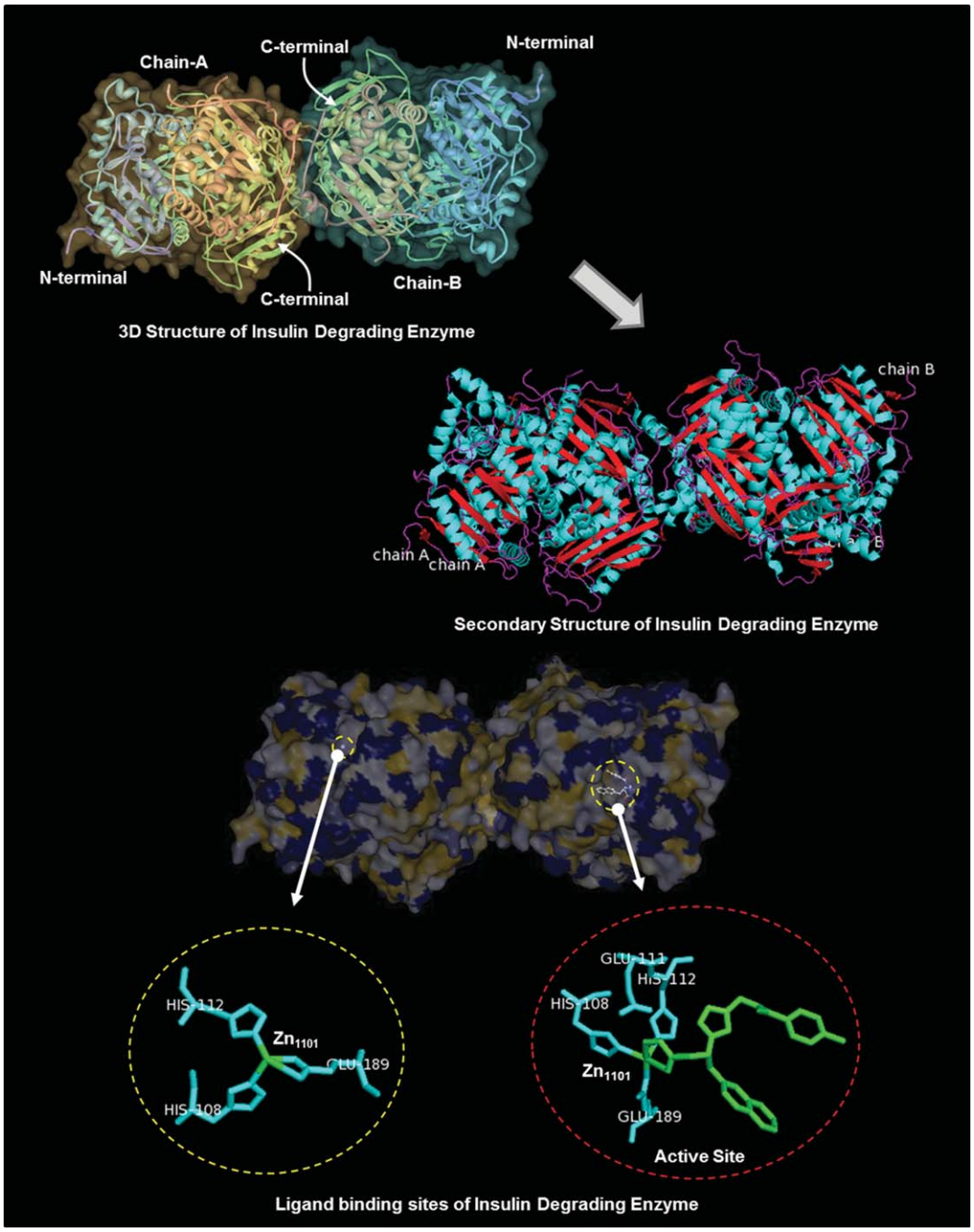


Fig. 5. 3D architecture of Insulin degrading enzyme (PDB ID: 4IFH). Secondary structure of protein has 44 helices (blue), 32 strands (red), and loops (pink) without any disulfide bridges. Ligand binding sites of IDE: Ligands are shown in green, while ligand binding residues are shown in blue (Helix forming residues). Chain A: Zinc metal binding residues- His108, His112, Glu189; Chain B: Zinc metal binding residues (His108, His112, and Glu189) and active site residue (Glu111).

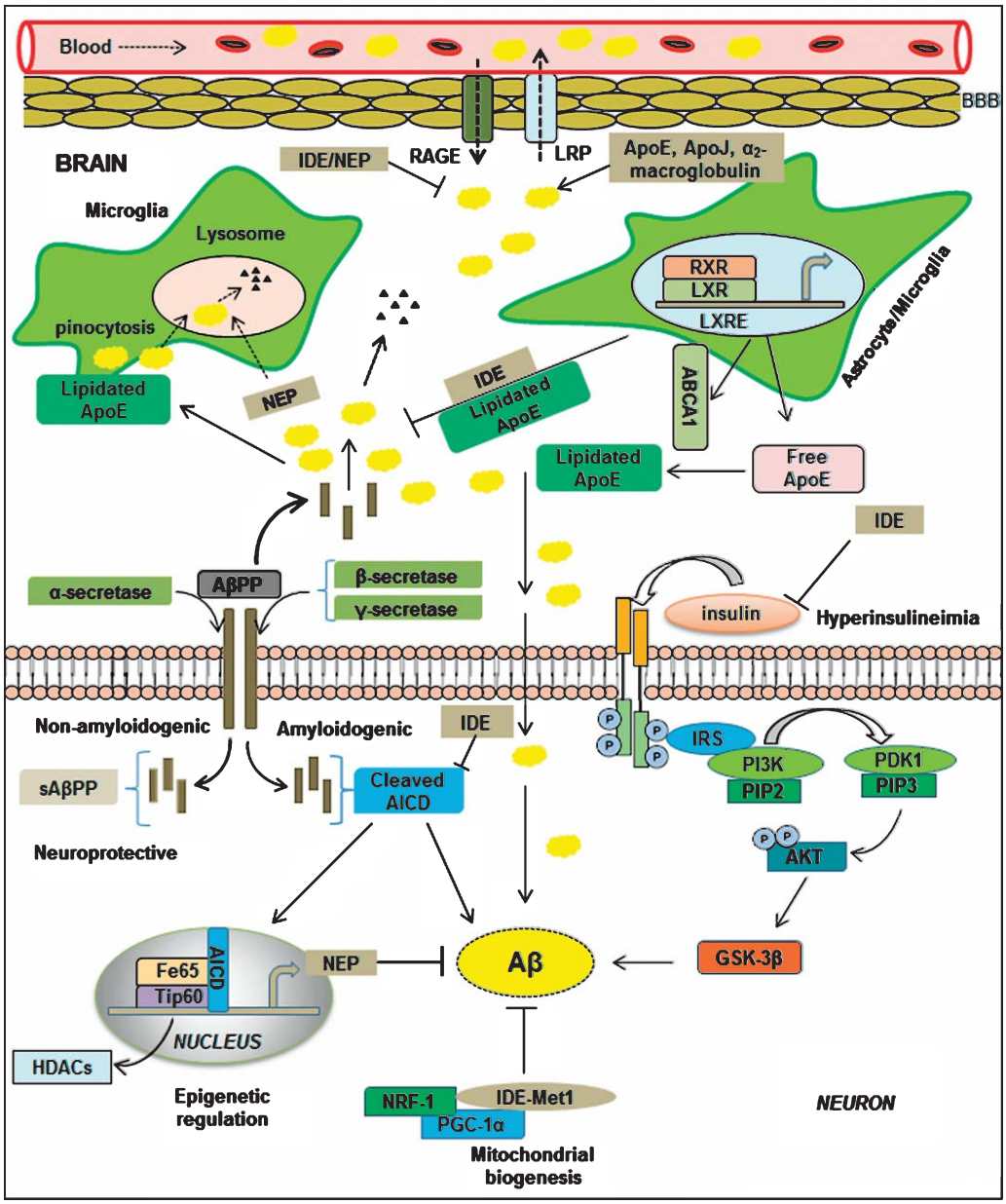


Fig. 6. Cross-talk between insulin degrading enzyme and neprilysin (NEP)-mediated Aβ degradation. Following Aβ aggregation via amyloidogenic pathway, simultaneously Aβ degradation is also critical for normal brain functionality. Here in this figure, IDE and NEP are playing a crucial role in order to reduce the toxic effect of Aβ. IDE is degrading AICD, insulin, and mitAβ, and also directs the extracellular degradation of Aβ together with lipidated ApoE, whereas NEP is epigenetically regulated by AICD raising its expression for Aβ clearance and also involved in intracellular Aβ degradation along with lipidated ApoE. Further, these enzymes are also participating in RAGE-mediated Aβ degradation inside the brain, while ApoE, ApoJ, and α_2-macroglobulin is assisting Aβ transportation outside the brain via LRP complex for degradation in liver.

INTEGRATIVE ROLE OF INSULIN DEGRADING ENZYME AND NEPRILYSIN IN AD THERAPEUTICS

In the case of AD, it is difficult with a single model to entirely mimic the disease progression in humans. Therefore, models are developed based on different proposed pathways for testing the pharmacodynamics of the drugs and also the biology of the disease and its development [124]. To date, extracellular accumulation of Aβ is found to be the main pathology involved in AD, thus reduction of Aβ deposition is the major

concern for neurobiologists. To achieve this goal, different strategies need to be formulated that could slow down the disease progression or even lead the way to discover its treatment. Such a method includes the degradation of Aβ with the action of proteolytic enzymes such as NEP and IDE [43]. Moreover, elevated concentration of Aβ has been observed in response to altered gene expression of NEP and IDE. In one study, overexpression of IDE and NEP in AβPP-transgenic mice was found to decrease Aβ levels in the brains of the transgenic mice as compared to the non-transgenic mice. Mice that expressed IDE showed 2-fold reduction in Aβ deposition, while mice that showed the 8-fold increase in NEP levels were free of Aβ deposits, thereby exhibiting its potential in reduction of lethal phenotype [125, 126]. These observations conclude that enhancement in the level of these enzymes would confer a protective effect against AD. Thus, supplying excess of degrading enzymes with the help of various approaches such as gene therapy or pharmacological drugs could be a potential therapy against AD [127–129]. The major hurdle impeding the development of novel therapies for AD is the ability to cross the BBB. However, this problem has recently been addressed in a number of ways as follows.

CURRENT STRATEGIES TO DELIVER INSULIN DEGRADING ENZYME AND NEPRILYSIN FOR THERAPEUTIC PURPOSES

At present, coupling of NEP with red blood cells is used to transport NEP from brain to plasma where it can effectively degrade Aβ. Further, lentiviral vector expressing NEP was fused with apolipoprotein B (ApoB) that binds with LDL-receptor to facilitate its transport to CNS and other parts of the brain, thereby decreasing Aβ levels in an AβPP-transgenic mice model [130, 131]. The reason behind this is its high cloning capacity and ability to form complex cassettes of lentiviral vector, and it is widely being used for long-term expression of NEP to reduce Aβ burden in the brain [132, 133]. Further, stereotaxic infusion of NEP-encoding viral vectors into the hippocampus has been shown to reduce Aβ burden in AD brain. However, a more competent and global delivery system is required to target widely distributed Aβ, and one such system is the adeno-associated viral vector (AAVV) that provides neuronal gene expression throughout the brain after peripheral administration; for instance, a single intra cardiac administration of the vector carrying NEP gene in an AD-mice model has been shown to elevate expression of NEP throughout the brain, thereby reducing Aβ oligomer formation [134, 135]. Another viral vector, Sindbis viral vector, has also been used to reduce Aβ burden in the brain where the NEP gene is inserted into the viral vector and then allowed to infect the neurons selectively and efficiently with elevated NEP levels [136, 137]. Moreover, intracranial viral vector delivery by injecting NEP directly into the brain has also been reported to reduce Aβ deposition, while intravenous infusion of a recombinant enzyme is a clinically desirable therapy [34]. However, earlier studies revealed the significance of NEP expressing viral vectors in reducing Aβ levels in AD brain, but it has several drawbacks regarding control of insert size, desired expression (short- or long-term), and their target cell type. Therefore, in order to overcome these drawbacks, recently recombinant soluble NEP expression vector from insect cells has been transfected into AD mice with the help of intracerebral injection, thereby ameliorating memory impairment in AD brain. In this way, protein therapy approaches might have been potential for development of alternative therapies for treatment of AD [138].

Another strategy for lowering Aβ formation is the downregulation of AβPP gene, which is possible with the newly emerging siRNA technology where siRNA against AβPP mRNA is used. These siRNA approaches have worked well in cell culture, but they lack enough potentiality to express their function inside the cells due to lack of efficient delivery systems. In order to achieve RNA interference effect, short hairpin RNAs (shRNA) is used in viral vectors such as retroviruses and adeno-associated viruses due to their good transduction efficiency along with the long-term expression of transgene in nervous system [139]. Another approach to facilitate enhanced clearance of Aβ, a replication-defective Herpes simplex virus (HSV) based vector has been developed that comprised of genes required for maintaining its latency state as well as transgene expression in the host. For instance, HSV-NEP vector is designed to express NEP that showed 5-fold reductions in Aβ levels, and this effect is found to be upregulated with the increased infection by viral vectors. Thus with the use of HSV vectors, the siRNA against AβPP can also be delivered into the host with the aim of lowering Aβ levels [140]. However, yet another strategy to eliminate Aβ peptide is the administration of auto-antibodies, where Aβ-cDNA expressing recombinant adeno-associated viral vectors (rAAV) is injected intramuscularly or

orally to induce auto-antibody production against Aβ [139, 141, 142].

Another major concern with the use of gene therapy in treating AD is the delivery of the gene into Aβ susceptible areas. This goal can be achieved by using monocytes, which are the major immune cells that migrate to the Aβ site. In this approach, genes can be transfected into bone marrow cells with a specific promoter that allows the expression of the genes in differentiated macrophages. For instance, $CD11b^{+}$ cells (monocytes) that are transfected with NEP, when injected twice a week in a transgenic mice model, stopped the amyloid deposition completely. The efficacy and safety of this method have been confirmed; therefore, this could be a more potent and permanent treatment for AD [143]. Further, evidence suggests a role of short-term neural stem cell (NSC) transplantation in ameliorating cognition in an AD transgenic mice model by improving endogenous synaptic connectivity. But,this approach has no longer the effect on the underlying neurofibrillary tangle and Aβ pathology. Therefore, combinatorial approaches of gene therapy are required for achieving long-term efficacy; for instance, in 3xTg-AD and Thy1-AβPP transgenic mice model, stem cell-mediated delivery of NEP showed significant reductions in Aβ pathology along with increased synaptic density. Remarkably, these Aβ plaque burdens are reduced not only in the hippocampus and subiculum adjacent to engrafted NSCs, but also within the amygdala and medial septum areas, that receive afferent projections from the engrafted region [144]. Another approach to deliver NEP is using human adipose tissue-derived mesenchymal stem cells (ADSCs) that release enzymatically active NEP carrying exosomes. Moreover, ADSC-derived exosomal delivery into neuroblastoma cells (N2a) resulted in the reduction of both secreted and intracellular Aβ levels [145]. Recently, a convection-enhanced delivery of NEP gene resulted into a 20-fold increase in NEP protein level with preserved enzyme activity in the striatum, thereby causing significant reduction in endogenous $A\beta_{40\text{-}42}$ levels [146, 147]. Most importantly, the delivery of insulin antibodies or insulin-inhibiting peptides into the brain can be a prominent therapeutic approach against toxic Aβ. However, fewer practical but augmented IDE levels in the CNS or disease prone site in the brain (via IDE infusion or gene therapy), would be of therapeutic interest [30]. Moreover, further investigation is to be needed to explore the gene therapy potential of IDE in the brain in order to nullify the toxic effect of Aβ. Since proper regulation of IDE and NEP is essential for their therapeutic applications, recently identified factors/agent controlling their expression level is discussed here.

RECENTLY IDENTIFIED THERAPEUTIC AGENTS REGULATING IDE AND NEP LEVELS IN THE BRAIN

Sex steroids are one of the therapeutic agents who ameliorate cognitive deficits in AD animal models by reducing Aβ levels in the brain due to elevated expression of IDE and NEP. For instance, administration of androgen receptor agonist (ACP-105) in association with estrogen receptor β (ERβ) agonist (AC-186) in male gonadectomized triple transgenic mice showed elevated levels and activity of IDE and NEP [148]. Another agent, nitric oxide synthase (NOS2) which is overexpressed in case of AD, was also found to be involved in the regulation of IDE and NEP. An *in vitro* study revealed that activity of IDE was inhibited by nitric oxide donor 'Sin-1', while NEP activity remains unaffected. However, an *in vivo* study showed downregulated activity of IDE in AβPP/PS1 mice, but not in AβPP/PS1/NOS2 (-/-) mice, signifying the role of NOS2 in preventing Aβ degradation through negative regulation of IDE. Thus loss of NOS2 activity would have been promising effect in positively triggering Aβ clearance in AD brain [149]. Recently, amyloid associated proteins, including ApoJ and ApoE, were found to modify Aβ uptake in human astrocytes where NEP expression is increased upon exposure to ApoE in combination with both Aβ preparations, i.e., Aβ-oligomer and Aβ-fibrillar, but NEP and IDE expression remain unaffected by exposure to Aβ-oligomeric or Aβ-fibrillar alone. It is important to note that Aβ alone indeed does not affect the astrocytic expression of IDE and NEP [102]. However, use of retinoic acid receptor (RARα) agonist has been reported to promote Aβ clearance by augmenting IDE and NEP activity in both microglia and diseased neurons [150]. Based on these observations, retinoic acid is a potential molecule, involved in the maintenance of synaptic plasticity of neurons, which may assist in keeping memory function intact.

Interleukin-34 (IL-34), a newly discovered cytokine, is found to overexpress IDE in microglia where it decreases oxidative stress without generating neurotoxic molecules, thus promoting the clearance of soluble oligomeric Aβ and preventing synaptic dysfunction and neuronal damage in AD [151]. Moreover, interleukin receptor-associated kinases (IRAKs; intracellular signaling molecules) have been reported

Table 5
List of agonists and antagonists regulating IDE and NEP expression level

S. No.	Agonists	Targets	Molecular Functions	References
1	DHA (Docosahexaenoic acid)	IDE	It is the most important fatty acid in the brain especially rich in the neurons and synaptosomes of the cerebral cortex. DHA significantly upregulates the expression of IDE in neural cells.	[163]
2	ATP (Adenosine triphosphate)	IDE	ATP facilitates the transition from the closed state to the open conformation. Biophysical analysis of ATP-induced conformational switch of IDE thereby showed the increased expression of IDE.	[164]
3	Valproic acid	NEP	Valproic acid attenuates the prenatal hypoxia-induced Aβ neuropathology, learning, and memory deficits via inhibiting the activation of histone deacetylase 1 (HDAC1), preventing the decrease in H3-Ace in the NEP promoter regions and reducing the down-regulation of NEP.	[165, 166]
4	Propranolol	IDE	It enhances the expression of IDE. Moreover, the expression of Akt, BDNF, and Tau hyperphosphorylation is decreased by propranolol treatment as shown by Tg2576 mice.	[167]
5	Neuropep-1	IDE & NEP both	It increases the level of IDE and NEP. However, Neuropep-1 treatment does not alter the expression of full-length AβPP, α-, β-, or γ-secretase.	[168]
6	Somatostatin (octreotide)	IDE	Somatostatin directly interacts with IDE. It binds to the active site of one IDE subunit, induces an enhancement of IDE proteolytic activity toward fluorogenic Aβ by another subunit.	[169, 170]
7	Imidazole-derived 2-[N-carbamoylmethyl-alkylamino] acetic acids	IDE	It acts as substrate-dependent modulators of IDE in Aβ hydrolysis.	[171]
8	Cysteine 904	IDE	Cysteine 904 is required for Maximal IDE Activity and Polyanion Activation.	[172]
9	BRI2 (British precursor protein)	IDE	BRI2 acts as a receptor protein that regulates IDE levels, and in turn influences AβPP metabolism.	[173]
10	Apomorphine (APO)	IDE	Apomorphine, a kind of dopamine receptor agonists responsible for promoting the intracellular Aβ degradation via activating IDE.	[174]
11	Leptin	IDE	Leptin enhances the expression level of IDE putatively by activating the Akt pathway.	[175, 176]
12	Suramin	IDE	Suramin increases the activity of the enzyme IDE by changing the turnover rate of the enzyme for its substrate.	[177, 178]
13	Estrogen	NEP	Estrogen positively regulates expression of NEP to promote degradation of Aβ and reduce the risk of AD.	[179]
14	Humanin (HN)	NEP	Humanin (HN), a 24-residue peptide act as a Neuroprotective factor, which shows anti-cell death activity against a wide spectrum of AD. It also increases the expression of NEP.	[180]
15	Trichostatin (TSA)	NEP	TSA treatment significantly enhances NEP expression by elevating the acetylation of histone H3 on NEP promoter.	[181]
16	5-aza-deoxycytidine (5-Aza-dc)	NEP	5-Aza-dc induces the demethylation of NEP gene and significantly increases its expression in a dose-dependent manner.	[181]
17	Ginsenoside Rg1 & Rg3	IDE&NEP both	It increases the intracellular level of both IDE and NEP in the hippocampus by upregulating PPARγ, thereby decreasing Aβ burden.	[182, 183]
18	Imatinib (Gleevec)	NEP	It is known tyrosine kinase inhibitors, which elevates AICD in H4 human neuroglioma cells and also increase the expression of NEP protein.	[184]
19	GW742	IDE & NEP both	A PPARdelta agonist reduces amyloid burden by enhancing the expression of IDE and NEP.	[185]

Table 5
(Continued)

S. No.	Agonists	Targets	Molecular Functions	References
20	EGCG	NEP	EGCG strongly increases the NEP activity, thus lead to Aβ degradation.	[186]
21	Curcumin	IDE & NEP both	It increases Aβ clearance by increasing both IDE and NEP activity and also prevents Aβ production by inhibiting PS2, a catalytic component of γ-secretase.	[187]
22	Resveratrol (RSV)	NEP	RSV significantly increases both the estradiol and NEP level that decrease Aβ deposition; by upregulation of estradiol level which consequently leads to increase the level of NEP, thus contribute to Aβ degradation.	[188]
23	Norepinephrine	IDE	Norepinephrine augments microglia to uptake and degrades Aβ peptides through upregulation of IDE.	[189]
24	Testosterone	NEP	Testosterone increases neuronal viability in cultured hippocampal neurons through the AR-dependent MAPK/ERK signalling pathway. Thus, it elevates the levels of NEP to facilitate Aβ clearance.	[190–192]
25	Androgens	NEP	Androgens increases NEP expression through the AR-dependent MAPK/ERK signalling pathway.	[193]
26	Nobiletin	NEP	It enhanced NEP activity both at the gene and protein level in time- and dose-dependent manner in SK-N-SH cells and thereby promoting Aβ clearance.	[194]
S. No.	Antagonists	Targets	Molecular Functions	References
1	Palmitic acid (PA)	IDE	PA treatment significantly reduces the expression level of IDE, an important protease responsible for the degradation of Aβ in neural cells.	[163]
2	Sevoflurane	IDE and NEP both	Sevoflurane alters the expression of receptors and enzymes involved in Aβ clearance, thereby reducing the levels of IDE and NEP in the brain.	[195]
3	Streptozotocin	NEP	It decreases the NEP activity in the hippocampus and cortex regions, thereby increasing Aβ level.	[196]
4	Thiorpan	NEP	Intracerebroventricular infusion of thiorphan, a NEP inhibitor raises cortical and cerebrospinal fluid (CSF) Aβ concentrations in the brain. It indicates that age-related decrease in NEP could lead to increased Aβ burden.	[197]
5	Tautomycetin	NEP	A specific inhibitor of protein phosphatase-1a, tautomycetin induces extensive phosphorylation of the Ser6 NEP intracellular domain, resulting into reduced cell surface NEP activity.	[153, 161]
6	Phosphoramidon	NEP	It acts as NEP inhibitor. Phosphoramidon induces a dramatic increase in Aβ levels, resulting into rapid plaque formation.	[198]
7	Nicastrin	NEP	Nicastrin deficiency drastically lowers NEP expression.	[199]
8	Ketamine	NEP	Ketamine suppresses the Aβ degradation of NEP by reducing p38 MAPK-mediated pathway activity.	[200]
9	Spinorphin	NEP	It is a heptapeptide, which inhibits dipeptidyl peptidases and angiotensin-converting enzyme activity as well as NEP activity.	[201, 202]
10	Sialorphin	NEP	It prevents spinal and renal NEP activity from breaking down its two physiologically relevant substrates, substance P and Met-enkephalin *in vitro*. It is a natural systemically active regulator of NEP.	[203]
11	Opiorphin	NEP	Opiorphin was a first NEP inhibitor isolated from saliva which had some pain-suppressive potency.	[204, 205]
12	Copper (Cu^{2+})	NEP	Copper down regulates NEP activity through modulation of NEP protein degradation.	[206]
13	Lead (Pb)	NEP	Pb causes both the over expression of AβPP and repression of NEP resulting in the build-up of Aβ.	[207]
14	Leptin	NEP	Leptin significantly decreases the expression of NEP through activation of ERK signalling cascade.	[208]

to trigger TLR signals that initiate the canonical pro-inflammatory signaling pathways, which further activate JNK-p38 and ERK-MAP kinases to generate reactive oxygen species. Therefore, evidence suggests that functional loss of IRAK4 promotes amyloid clearance mechanisms by raising IDE expression [152]. In a recent study, protein phosphatases have been implicated in the reduction of kinase-mediated Aβ deposition; for instance, elevated Aβ levels in AD patients have been reduced in response to activated protein phosphatase-1a that dephosphorylate NEP and result in enhanced cell-surface NEP activity [153]. Likewise, another candidate, mitochondria-targeted antioxidant catalase, has been identified that possesses a protective role against Aβ toxicity and prevents abnormal AβPP processing through increased IDE and NEP activity [154].

A recent study revealed that human placenta amniotic membrane-derived mesenchymal stem cells, which are known for their potent immune-adulatory and paracrine effects, also have a pivotal role in improving spatial learning via increasing NEP and IDE expression in the brain [155]. Furthermore, using human-induced pluripotent stem cell-derived macrophage cells harboring NEP2 demonstrated decreased levels of Aβ in the 5XFAD mice model [156]. Moreover, neural stem cells carrying NEP ameliorated AD pathology by restoring synaptic density and reducing Aβ load in transgenic mice models of AD via 3xTg-AD and Thy1-AβPP [157]. Recently, it has been identified that various drugs, e.g., MK8931, AZD3293, E2609, and TPP488, have been used to reduce Aβ burden in the brain by triggering endogenous expression of IDE and NEP. These drugs act as an inhibitor of different signaling molecules, mainly targets on BACE1, BACE2, and RAGE molecule. These signaling molecules are basically involved in AβPP cleavage and their processing that lead to increased Aβ concentration in the brain. Furthermore, MK8931, AZD3293, E2609, and TPP488 treatment have been noticed, signifying their major contribution in a dose-dependent attenuation of Aβ levels in plasma and cerebrospinal fluid [158–161]. Besides therapeutic potential of NEP and IDE, their elevated levels have also been found associated with several side effects; for instance, cAMP-responsive element-binding protein (CREBP) mediated transcription caused age-dependent axonal degeneration, and it is also involved in the progression of various cancers [65, 162]. Therefore, in order to escape the side effects, we need to adopt the combinatorial therapeutic approach by using their agonists and antagonists for targeting Aβ toxicity in the brain. Such agonists and antagonists of IDE and NEP have been elaborated in Table 5.

CONCLUSIONS

The biology behind Aβ deposition in the brain and the clearance mechanism is quite complicated, and thus much attention has been given in the past few decades to understand these phenomena and their significance in AD. The etiopathology of AD is caused by accumulation of toxic Aβ peptides that occurred due to several factors, mainly associated with proteolytic cleavage of AβPP by β-secretase and γ-secretase, that lead to the formation of AICD and toxic Aβ. Protective enzymes such as IDE and NEP are found to play a crucial role in degradation of amyloidogenic or toxic Aβ peptides in the brain, thus signifying their therapeutic potential. Both IDE and NEP are metalloendopeptidases, which require Zn^{2+} ion as a cofactor for their catalytic activity. Multiple sequence analyses of both IDE and NEP [*Homo sapiens*] have revealed a sequence identity with *Macaca mulatta* of 99%. Sequential analysis has also revealed the presence of different post-transcriptional modification sites, for instance, protein kinase-C phosphorylation sites, tyrosine kinase phosphorylation sites, and casein kinase II phosphorylation sites, depicting their role in functional regulation of IDE and NEP. Further, structural analysis of both IDE and NEP has shown the presence of amino acids (His, Glu, and Asp) at their zinc binding motif and active site, which are responsible for their proper functions. Importantly, closely associated putative partners of both IDE and NEP have been identified that have almost similar functions and sequence identity with this peptidase.

In addition to *in silico* study of IDE and NEP, its elevated expression in the brain is important for rendering the toxic effect of Aβ. For that, more competent and global delivery systems like lentiviral vector, adeno-associated viral vector, siRNA, and recombinant gene therapy based delivery of IDE and NEP is required to target widely distributed area in the Aβ affected brain. Furthermore, a convection-enhanced delivery system is also used to transfer these enzymes to Aβ laden areas in the brain to reduce its toxicity. Additionally, using different agonists and antagonists based combinatorial therapeutic approach has also been shown for proper homeostatic regulation of both IDE and NEP, as high levels of these peptidases cause some side effects. Moreover, any changes in the levels of IDE and NEP cause accumulation of Aβ which is highly toxic to the cell,

because the levels of these two peptidases are altered in an age-dependent manner. In older age due to its downregulation, it does not influence the proteolytic degradation of Aβ and thus accumulation of toxic Aβ hampers the normal functionality of neuronal cells and therefore causes changes in the morphology of the neurons, memory loss, and consequent cell death.

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Role of Oxidative Stress, ER Stress and Ubiquitin Proteasome System in Neurodegeneration

Abstract

Neurodegenerative disorders (NDDs) are progressive and chronic disorders characterized by destruction of neurons in sensory, motor and cognitive systems. Free radical's accumulation, oxidative stress, ER stress and a dysfunctional ubiquitin proteasome system can regulate prognosis in NDDs. Oxidative trauma in the brain can result from high rate of oxidative metabolism in contrast to the diminished functional levels of the antioxidant enzymes responsible for detoxification. Endoplasmic Reticulum (ER) advocates a degree of control on cellular parameters such as proper protein folding, posttranslational modification and subsequent protein trafficking in order to maintain normal cellular homeostasis. However, an abnormal ER functioning can lead to loss of integrity of the ER thus resulting in ER stress. In addition to this impairment in the ubiquitin proteasome system (UPS) machinery results in the accumulation of toxic proteins in the brain thus resulting in severe neuronal trauma and subsequent damage. This review explores the disease critical interactions and roles of three critical NDD determinants viz. oxidative stress, ER stress and UPS dysfunction in neurodegenerative conditions.

Keywords

NDD; Free radicals; ROS; Oxidative stress; ER stress; UPS; E3-ligases; Mitochondrial dysfunction

Review Article

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Abbreviations

NDD: Neurodegenerative Disorders; ER: Endoplasmic Reticulum; UPS: Ubiquitin Proteasome System; ROS: Reactive Oxygen Species; AD: Alzheimer's Disease; PD: Parkinson's Disease; MS: Multiple Sclerosis; HD: Huntington's Disease; ALS: Amyotrophic Lateral Sclerosis; PS: Presenilin; APP: Amyloid beta (A4) Precursor Protein; MARK1: Microtubule Affinity-Regulating Kinase 1; SOD-1: Superoxide Dismutase 1; VAMP : Vesicle-Associated Membrane Protein; ALS2: Amyotrophic Lateral Sclerosis 2; DCTN1: Dynactin 1; FUS : FUS RNA binding protein; TDP- 43: TAR DNA binding Protein; PERK: Phosphorylation of the ER stress Kinases; IRE 1: Inositol-Requiring Enzyme 1; SCA: Spinocerebellar Ataxia; GSK: Glycogen Synthase Kinase; ASK1: Apoptosis Signal-Regulating Kinase 1; JNK: c-Jun NH2-terminal kinase; Keap1: Kelch-like ECH-associated protein 1; MGRN1: Mahogunin Ring finger 1, E3 ubiquitin protein ligase; MYCBP2: MYC Binding Protein 2, E3 ubiquitin protein ligase; UHRF2: Ubiquitin-like with PHD and Ring Finger domains 2; ZNRF1: Zinc and Ring Finger 1, E3 ubiquitin protein ligase; NEDD4: Neural precursor cell Expressed, Developmentally Down-regulated 4, E3 ubiquitin protein ligase; NEDD4L: Neural precursor cell Expressed, Developmentally Down-regulated 4-like, E3 ubiquitin protein Ligase; HECTD2: HECT domain containing E3 ubiquitin protein ligase 2; PJA2: Praja ring finger 2, E3 ubiquitin protein ligase; RNF19: Ring finger protein 19A, RBR E3 ubiquitin protein ligase; HECTD1: HECT domain containing E3 ubiquitin protein ligase 1; MULAN: Mitochondrial E3 Ubiquitin protein Ligase 1; HACE1: HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1; TRIM13: Tripartite Motif containing 13; AIMP2: Aminoacyl tRNA synthetase complex-Interacting Multifunctional Protein 2; NRF2: Nuclear Factor, erythroid 2-like 2; DVL1: Dishevelled Segment polarity protein 1; MYC: v-MYC avian Myelocytomatosis viral oncogene homolog; TSC2: Tuberous Sclerosis 2; FBXO45: F-Box protein 45; PCNP: PEST Proteolytic Signal Containing Nuclear Protein; SMAD2: SMAD family member 2

Introduction

Neurodegenerative disorders (NDDs) are characterized by the gradual and progressive loss of neurons and neuronal death that ultimately leads to deficient nervous system functioning. It can result due to diverse factors such as oxidative stress, ER stress, mitochondrial dysfunction, impaired ubiquitin proteasomal system and several other determinants such as endocrine conditions, gender, poor education, inflammation, stroke, smoking, hypertension, diabetes, infection, head trauma, depression, tumors, vitamin deficiencies, immune and metabolic conditions, chemical exposure, accumulation of reactive oxygen species (ROS), loss of mitochondrial membrane potential, and ATP depletion. The two hit hypothesis of neurodegeneration states that neuronal cells that have been subjected to a severely stress once, becomes more vulnerable to the negative impact of a second hit and the effect of the toxicity of both the hits of severe stress may be synergistic in nature. Most common neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Schizophrenia, Amyotrophic lateral sclerosis (ALS) and Multiple Sclerosis (MS) [1-5].

Oxidative stress plays a critical role in the progression of several age related brain disorders. Severe oxidative trauma to the neurons can result in neuronal dysfunction and death.

However, the neuronal cells are equipped with an arsenal of protective mechanism to prevent the damaging effects of oxidative stress on neuronal integrity and homeostasis. The removal of aberrantly functioning proteins by proteolysis and the synthesis of new and protective counterparts are critical during periods of continuous oxidative trauma [6]. Reactive oxygen species (ROS) can result in oxidative stress and subsequently lead to mitochondrial dysfunction. Moreover, disturbed equilibrium between pro-oxidant/antioxidant homeostasis can generate ROS and free radicals which are detrimental for neurons. ROS in turn modulates the functionality of antioxidants and biomolecules thus leading to neuronal dysfunction and advances the brain towards progressive neurodegeneration [7-10].

Endoplasmic reticulum (ER) mediated stress on the other side results from disturbances in the structural integrity and function of the ER, thus leading to the accumulation of misfolded proteins and deviations in the calcium homeostasis. The endoplasmic reticulum (ER) acts as protein quality control in the secretory pathway to prevent protein misfolding and aggregation. Under conditions of stress, the ER mediated machinery can reestablish homeostasis by sophistically regulating various transcriptionally and translationally mediated signaling networks and proteins [11]. The normal ER response is depicted by reduction in damaged proteins levels, caused by translational attenuation, induction of ER chaperones and misfolded proteins proteolysis. However, under prolonged or provoked ER stress ambience, can lead to the activation of apoptotic pathways resulting in neuronal death.. Therefore, ER stress situation and remains a subject of curious debate involving the pathogenesis of common NDDs such as Parkinson's disease (PD) and Alzheimer's disease (AD) [12].

Improper functioning of the Ubiquitin proteasome system (UPS) under conditions of severe ER and oxidative trauma leads to the derogatory accumulation of damaged and misfiring stress consistent proteins. The UPS in collaboration with chaperones and co-chaperones constitute the regulatory mechanism responsible for neuronal quality control and survival. In addition, UPS can also operate as machinery for protein quality control and degradation in conjunction with autophagy, Dysfunctional UPS has been held capable in various NDDs and recent reports on Drosophila suggested that the role of UPS in protein turnover is essential for maintaining axon guidance, synaptic function and growth, axon pruning, and neuronal maintenance [13]. Under the ambit of this review we have made an attempt to explore the role of oxidative stress, ER stress and UPS dysfunction respectively in neurodegenerative conditions. This interaction shall than be crucial in embellishing the development of potential neurotherapeutics.

Role of Oxidative Stress in Neurodegeneration

Oxidative stress (OS) condition in the brain results from imbalance between ROS and the body's detoxification mechanism, which results in accumulation of ROS and subsequent neuronal damage. Hence, the outcome of oxidative stress on neuronal cells depends upon the ability of the cell to maintain oxidative homeostasis. High stress levels can cause ATP depletion, necrosis and prevent apoptotic cell death [14].

Any disproportion in the usual redox state can result in toxicity via the activation of peroxides and free radicals which in turn damages lipids, proteins and cellular DNA. A mammalian cell as a consequence of mitochondrial aerobic respiration generates superoxide radical. Superoxide is sequentially reduced to hydroxyl radicals and hydrogen peroxide that cause severe traumatic injury to the DNA thus leading to mutations, which might be causative factors leading to severe neurodegeneration [15].

Reactive oxygen species (ROS) also plays a discrete role in cell signaling, by a mechanism known as redox signaling. In order to sustain proper cellular homeostasis, a balance must be reached between ROS production and consumption. Therefore, it is obvious that free radicals need to either be reduced and detoxified by converting them into metabolically nondestructive molecules or be neutralized right after their generation. Any aberration in the cellular antioxidant defense system, which protects the neurons from free radical assaults, therefore can lead to neurodegenerative conditions and aging [16].

Brain is the most metabolically active organ of the body that including the spinal cord comprises the central nervous system (CNS), which even in resting condition consumes an estimated 20-22% of the total oxygen uptake. In addition, during active state the brain oxygen demand considerably rises in order to establish normal physiological homeostasis. Blockage or oxygen deprivation can lead to severe and irreversible injuries to the neurons. Oxygen consumption in the brain of oxygen results in production of free radicals and higher oxygen levels in brain leads to even higher concentration of reactive oxygen/nitrogen species. However, in spite of the fact that brain has higher necessity for oxygen, it is relatively deficient in the enzymes capable of metabolizing a number of these toxic oxygen-based reactants to harmless residues. In contrast, CNS is highly enriched with polyunsaturated fatty acids and toxic oxygen derivatives oxidizes these polyunsaturated fatty acids [17,18]. This then makes the neurons more vulnerable to oxidation related damages and the role of the cellular detoxification machinery in these conditions is vital.

Oxidative stress has major impact on several NDDs such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) In AD, oxidative stress is associated with a number of critical events which ultimately influence amyloid precursor protein (APP) processing. Tau modification leads to increased brain toxicity and as a result of oxidative stress, APP and Tau processing is altered via activation of different signaling pathways. In general most of the AD cases are late onset and sporadic although there are approximately 10-15% familial AD (FAD) cases. Mutation in three genes namely, APP, Presenilin 1 and 2 (PS1, PS2) can potentially lead to FAD. During normal physiological state proteolytic cleavage of APP is commenced by α-secretase followed by γ-secretase mediated second cleavage to yield non amyloidogenic fragments [19]. However, mutations in APP results in an altered proteolytic processing where α- secretase is replaced by β-secretase (BACE1), followed by γ-secretase

mediated cleavage in order to yield amyloidogenic Aβ42 which aggregates as insoluble plaques. Widespread cell culture studies have revealed Aβ42 to have toxic effect on brain and can emanate cell death via apoptosis [20]. The hyperphosphorylation of tau protein, by various kinases such as MARK, MAPK and GSK-3αβ, results in the formation of paired helical filaments (PHFs), which further combine to form insoluble NFTs [21]. Abnormal hyperphosphorylation of tau is indicative of both an abnormal activation of kinases and decreased phosphatase activity [22]. Experiments on Pin1 knockout mice illustrates a rise in amyloidogenic APP processing thus increasing the levels of Aβ42 and additionally also display tau hyperphosphorylation thus leading to behavioral deficits, motor and neuronal degeneration [23].

Oxidative can modulate pathogenesis in Parkinson's. PD is the most common neurodegenerative disorder and is clinically demarcated by bradykinesia, progressive rigidity and tremor. Like all other neurodegenerative disorders determinants such as environmental factors, mitochondrial dysfunction, oxidative damage, and genetic predisposition together play a crucial role in both sporadic as well as familial PD [24]. Neurotoxic compounds, such as N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or its active derivative, MPPT and 6-hydroxydopamine (6-OHDA) can provoke oxidative stress, impair mitochondrial respiration and energy metabolism which in turn leads to neurodegeneration. Postmortem tissues from PD patients have revealed a significant insight into the failure of complex I in the substantia nigra. Complex I is involved in the mitochondrial electron-transport chain, and 30-40% decrease in activity may be the central prognosis of sporadic PD [25]. The decreased activity of complex I could be the result of self-inflected oxidative damage, under production of certain, complex I subunits, and may be due to complex I disassembly [26]. Immunocytochemical confirmation of protein glycation and nitration in substantia nigra region of human PD brain revealed oxidative damage to DNA and protein resulting from persistent oxidative trauma [27].

Huntington's disease (HD) is autosomaly inherited and is characterized by progressive cognitive impairment, choreiform movements, psychiatric disturbances and loss of long projection neurons, resulting in atrophy of the caudate nucleus, globus pallidus, and putamen [28]. HD mutation is an extension of the CAG trinucleotide repeat inside exon 1 of the huntingtin (*HTT*) gene, the exact role of which is unknown [29]. CAG triplet codes for glutamine expansion and upon mutation, presents a polyglutamine tract at the N-terminus, results in a conformational change of the protein, which eventually results in abnormal protein-protein interaction. Mutant HTT presents a dominant "gain of function" to the protein, due to the stretched polyglutamine segment, which finally leads to neurodegeneration. Empirical evidences suggest that the mitochondrial metabolic defect resulting in impaired energy metabolism may be the consequences of HTT gene expansion [30]. Altered mitochondrial energy metabolism raises the production of free radicals thus resulting in severe neuronal trauma. Mitochondrial dysfunction is a critical hallmark in the pathogenesis of PD. The activity of mitochondrial complexes I, II, III and IV is significantly altered during HD pathogenesis. Biochemical studies of HD brain tissue have reported defects in the caudate and decreased activities of complex II and III activity. However, no such deviation was observed with complex I or IV [31].

Amyotrophic lateral sclerosis (ALS) is clinically identified by progressive atrophy, weakness, and spasticity of muscle tissue. ALS is characterized as an adult-onset neurodegenerative disease reflecting the degeneration of upper and lower motor neurons in the spinal cord, cortex, and brainstem [32]. Mutations in the ubiquitous enzyme; Cu/Zn-superoxide dismutase (SOD-1), accounts for upto 5-20% all of major genetic defect in ALS. In addition to SOD-1 gene few other genes such as VAMP-associate protein B (VAPB), Alsin (ALS2), Dynactin (DCTN1), fused in sarcoma protein (FUS), TAR DNA-binding protein-43 (TDP-43), and lipid phosphatase FIG4 (FIG4) can also contribute to ALS pathogenesis. Postmortem tissues from ALS patients have clearly revealed that oxidative stress is the main causative factor that contributes to accumulation of oxidative damage to lipids, proteins, and DNA thus suggesting a direct role in ALS progression [33,34].

Role of ER Stress in Neurodegenerative Conditions

Endoplasmic reticulum (ER) is an imperative organelle responsible for the post-translation modification, proper folding, and transport of nascent proteins to target destinations. Loss of ER integrity results in ER stress and may be established due to changes in the calcium homeostasis within the ER and due to the accumulation of unfolded proteins. ER stress plays a crucial role in several signaling cascades including the unfolded protein response (UPR) which counteracts the effects of the original stress [35,36]. The activity of the ubiquitin proteasome system (UPS) is significantly misregulated in these stress conditions and leads to protein aggregates and other toxic product accumulation thus leading to brain damaging conditions [37,38]. Furthermore, intracellular ER calcium concentration and its release from the ER play a significant role in controlling neuronal death [39].

As discussed earlier AD is a neurodegenerative disease characterized by the progressive loss of cognitive functions and memory loss. ER stress and an altered calcium homeostasis have major impact on severity of AD pathogenesis [40,41]. Brain tissue from AD patients reports an alteration in calcium metabolism and subsequent neurodegeneration. Neurons containing NFTs shows an increase in the levels of free and protein bound calcium as compared to tangle free neurons. In addition to change in the level of calcium ion due to ER stress, an alteration in APP or PS proteins activity also can define AD prognosis [42]. PS1 and PS2 proteins are the major catalytic components of the γ-secretase complex that facilitates the intramembranous cleavage of APP. ER stress mutations can cause a change in the pattern of APP processing in the affected neurons and as a result increase the amount of the toxic Aβ1-42 peptide. PS1 and PS2 are ER transmembrane proteins that are richly expressed by the brain neurons and which facilitates a linkage between AD and ER stress. ER stress related alteration in PS1 activity demonstrates altered calcium

homeostasis, increased production of Aβ peptides, and enhanced apoptotic sensitivity. Mutant PS1 attaches to and restrains the ER kinase, IRE1 which senses the gathering of misfolded proteins in the ER lumen. IRE also triggers the downstream signals to mediate the transcription of the ER chaperone, BiP [43].

Cultured neuronal cells, including dopaminergic neurons, reveals that neurotoxic compounds such as N-methyl-4-phenyl-1,2,3,6-tetrahydroyridine or its active derivative, MPPT and 6-hydroxydopamine (6-OHDA) can elicit ER stress and activate a number of genes such as the ER chaperones and other machinery of the UPR for instance the transcription factor, CHOP/Gadd153. In addition it can also lead to the phosphorylation of the ER stress kinases, PERK and IRE [44,45]. Thus, ER stress in combination with abnormal protein degradation can contribute to the pathophysiology of NDDs.

Human inherited neurodegenerative disorders such as Huntington's disease (HD), dentatorubral-pallidoluysian atrophy, spinobulbar muscular atrophy, and six spinocerebellar ataxias (SCA 1, 2, 3, 6, 7 and 17) are caused due to expanded polyglutamine (polyQ) repeats in the brain. In cultured cells, transgenic animals and in human post-mortem brain tissue these disorders are significantly characterized by aggregation of intracellular protein aggregates and selective neuronal death [46]. Additionally, in HD the mutant Huntingtin gene can also have an effect on the calcium metabolism in the cell and sensitize the IP3 receptors in the ER [47].

Evidence about the role of ER stress in polyQ diseases approaches from studies showing the colocalization of polyQ fragments with various molecular chaperones viz. Hsp70 and Hsp40 that are induced during ER stress. Drosophila overexpression of Hsp70 restrains polyQ toxicity. This Hsp mediated effect has also been observed in a few, but not all mouse models of polyQ diseases [46]. SCA3 polyQ fragments also triggers ER stress mediated neuronal cell death, as shown by the activation of PERK, IRE1 and the stimulation of CHOP/Gadd153 and BiP/Grp78. However, this effect is mainly due to impairment in the interaction between the ER and the UPS. Another study reveals that deficient mouse embryonic fibroblasts show activation in the apoptosis signal-regulating kinase 1 (ASK1) which is indispensable for polyQ induced ER-mediated cell death [48]. Moreover, ASK1 forms a complex with TRAF2 and IRE proteins at the ER and consequently trigger downstream signals, such as the c-Jun NH2-terminal Kinase (JNK) [49].

Transgenic ALS mice and human samples reports intracellular cytoplasmic inclusions in motor neurons. In ALS mice these contain deposits of SOD1 and ubiquitin. The aggregates appear prior to the first appearance of disease symptoms. Although the significance of inclusion bodies in ALS is not clear yet it has been attributed to act as a neurotoxin and inhibits critical cellular functionalities [50]. Dorfin is one such ubiquitin E3 Ligase which play a crucial role in ALS mediated neurodegeneration. In ALS infected neurons, mutant SOD1is degraded by such ubiquitin E3-ligase through the UPS. Mutant a-synuclein and aggregated SOD1 in combination with other proteins can alter the function of the UPS and also affects the motor neurons. Although, motor neurons are not the only cell type targeted in ALS. The role of glial neuron and glial cells interactions at some stage in development of the disease suggests that the increase in ROS production combined with ER and oxidative damage to crucial proteins and other cell machinery may play a role in ALS pathogenesis [51].

Glutamate metabolism is associated with prolonged stimulation of excitatory amino-acid receptors and results in increased intracellular calcium levels, which can easily damage the integrity and functional aspects of mitochondria and the ER. This then result in cleavage of caspase-12 in the spinal cord of transgenic ALS mice. Caspase-12 activity and cleavage in the ALS mice may be due to the activity of the calcium dependent enzyme, Calpain. Caspase-12 acts as substrate for calpain in some cells including neurons. Other biomarkers for ER stress, Bip/Grp78 function is also altered in the ALS mice [52]. Furthermore, evidence for ER stress in ALS comes from studies showing an increase in Bip/Grp78 level in spinal motor neurons of transgenic ALS mice proceeding to onset of motor symptoms. It has been also accounted that mutant SOD1is associated with ER stress, but not wild type SOD1 [53]. These findings provide credence to the fact that ER stress is part of the mechanism by which mutant SOD1 contributes to ALS related motor neuron degeneration.

UPS and E3 ligases in Neurodegeneration

Dysfunction of the ubiquitin proteasome system is one of the major events that lead to the progression of neuronal loss. An in vivo report suggests that oxidative stress is caused directly by neuronal proteasome dysfunction in the mammalian brain [13,54,55]. The UPS plays a vital role in regulated degradation of cellular proteins under diverse physiological conditions. Aggregation of misfolded proteins has been attributed in the progression of various neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). Ubiquitin E3 ligases are key regulators involved in mediating the proteasomal degradation of misfolded proteins in the endoplasmic reticulum (ER), as a result protecting neurons against oxidative stress, mitochondrial dysfunction and ER stress [56].Furthermore, Ubiquitin proteasome system can crtically modulate the level of proteins in cells, and robustly control cellular mechanisms. Aberration in UPS function in susceptible neurons results in protein aggregation, increased, oxidative stress, ER stress, and ultimately neuronal death. Conversely, neurons depend on the proper functioning of E3 ligases and UPS to maintain neuronal homeostasis [57]. Table 1 highlights the prospective role E3 ligases in neurodegeneration.

Conclusion

The future of neurodegenerative disorders depends on the researchers' ability to adjust actions to circumstances and have a clear projection relating to the aberrant mechanisms that ultimately decides the fate of the neurons and henceforth degeneration. Despite tremendous advancement in the field of neurobiology, still the future of such therapies hangs on torrid balance and deceptive hopes Neuronal damage is caused due to free radical's accumulation, oxidative stress and ER stress.

Table 1: E3 ligases in the brain and their functional prospect in neurodegeneration.

E3 Ligase	Substrates	Functional Significance	References
Keap1	Nrf2	Involved in degradation of Nrf2. disruption of protein degradation systems and sustained activation of the Keap1-Nrf2 system occur in the AD brain.	Tanji et al. [58]
PARK2	AIMP2	Parkin is an E3 ubiquitin ligase that has been shown to be a key regulator of the autophagy pathway. Although, mutations in Parkin results into Parkinson's Disease.	Segura-Aguilar et al. & Imam et al. [59,60]
HECW1	DVL1, p53, and mutant SOD1	NEDL1 is another name of HECW1. It mainly interacts with p53 and the Wnt signaling protein DVL1, and may play a critical role in p53-mediated cell death in neurons.	Li et al. [61]
HUWE1	TopBP1, N-Myc, C-Myc, p53, Mcl-1	HUWE1 regulates neuronal differentiation by destabilizing N-Myc, and also modulates p53-dependent and independent tumor suppression via ARF. It is also known as Mule. HUWE1 is a HECT domain E3 ubiquitin ligase which involves in degradation of Mcl-1 and thus regulates DNA damage-induced apoptosis.	Zhong et al. [62]
MGRN1		Involved in melanocortin signaling. Loss of mahogunin function leads to neurodegeneration and loss of pigmentation, and also has mechanism of action in prion disease.	Perez-Oliva et al. [63]
MYCBP2	TSC2, Fbxo45	MYCBP2 associates with Fbxo45 to play a crucial role in neuronal development. MycBP2 is an E3 ubiquitin ligase also known as PAM. MycBP2 also modulates the mTOR pathway through ubiquitination of TSC2.	Han et al. [64]
UHRF2	PCNP	UHRF2 ubiquitinates PCNP and has been shown to play a role in degradation of nuclear aggregates containing polyglutamine repeats mediated Neurodegeneration. UHRF2 is also known as NIRF. UHRF2 is a nuclear protein that may regulate cell cycle progression through association with Chk2.	Mori et al. [65]
ZNRF1		Highly expressed in neuronal cells. ZNRF1 is found in synaptic vesicle helpful in neuronal transmissions and plasticity. It also contains a RING finger motif, which expression is up regulated in the Schwann cells mediated nerve injury.	Araki and Milbrandt [66] & Saitoh and Araki [67]
NEDD4		Highly expressed in the early mouse embryonic central nervous system. It down regulates both neuronal voltage-gated Na^+ channels and epithelial Na^+ channels in response to increased intracellular Na^+ concentrations.	Goulet et al. [68]
NEDD4L	Smad2	It also highly expressed in the early mouse embryonic central nervous system. NEDD4L negatively regulates TGF-β signaling by targeting Smad2 for degradation.	Gao et al. [69]
HECTD2		HECTD2 is a likely E3 ubiquitin ligase and may act as a vulnerable gene for neurodegeneration especially in prion disease.	Lloyd et al. [70]
PJA2		Expressed in neuronal synapses. The exact role and substrates of PJA2 are unclear.	Yu et al. [71]
RNF19	SOD1	RNF19 is also known as Dorfin. Accumulation of mutant SOD1 results into ALS disease. RNF19 ubiquitinates mutant SOD1 protein, causing less neurotoxicity in brain.	Sone et al. [72]
HECTD1		HECTD1 is required for normal development of the mesenchyme and neural tube closure.	Zohn et al. [73]
MULAN	mnd2	Involved in degradation of mnd2. mnd2 causes neuromuscular disorder due to loss of Omi/HtrA2's protease activity.	Cilenti et al. [74]
HACE1	NRF2	HACE1 plays a crucial in the NRF2 mediated antioxidative stress response pathway and also involved in HD pathogenesis.	Rotblat et al. [75]
CUL4	TSC2	It promotes proteasomal degradation of TSC2. As a result, Tnfaip8 l1/Oxi-β competes with TSC2 to bind FBXW5, increasing TSC2 stability through preventing its ubiquitination in PD progression.	Ha et al. [76]
TRIM13		Involved in regulation of ER stress induced cell death. However, the expression of TRIM13 sensitizes cells to ER stress induced neuronal cell death.	Tomar et al. [77]
MGRN1		Over expression of MGRN1 protects against cell death mediated by ER and oxidative stress and also interacts with Cytosolic Hsp70. Lack of MGRN1 functionalities are the hall mark of age dependent spongiform disease in the brain.	Chhangani and Mishra [78]
NEDD4-1	FOXM1B	Up regulated in cultured neurons in response to various neurotoxins, including, hydrogen superoxide, and zinc via transcriptional activation likely mediated by the reactive oxygen species. A level of the insulin-like growth factor receptor (IGF-1Rβ) is also maintained due to up regulation of NEDD4-1.	Kwak et al. [79]
APC/C		Involved in cell cycle progression in proliferating cells, plays a significant role in post-mitotic neurons. APC/C-activating cofactor, Cdh1, is also helpful for the function of APC/C in neuronal survival.	Almeida [80]

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In addition, dysfunctional of ubiquitin proteasome systems can also regulate prognosis in NDDs. These factors can cause an imbalance between cellular-antioxidant defence and reactive oxygen species concentration. Further research is therefore needed in order to make bio molecule based neurotherapeutics a blatant reality in conditions of neurodegeneration.

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Review

Stress-Induced Synaptic Dysfunction and Neurotransmitter Release in Alzheimer's Disease: Can Neurotransmitters and Neuromodulators be Potential Therapeutic Targets?

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Abstract. The communication between neurons at synaptic junctions is an intriguing process that monitors the transmission of various electro-chemical signals in the central nervous system. Albeit any aberration in the mechanisms associated with transmission of these signals leads to loss of synaptic contacts in both the neocortex and hippocampus thereby causing insidious cognitive decline and memory dysfunction. Compelling evidence suggests that soluble amyloid-β (Aβ) and hyper-phosphorylated tau serve as toxins in the dysfunction of synaptic plasticity and aberrant neurotransmitter (NT) release at synapses consequently causing a cognitive decline in Alzheimer's disease (AD). Further, an imbalance between excitatory and inhibitory neurotransmission systems induced by impaired redox signaling and altered mitochondrial integrity is also amenable for such abnormalities. Defective NT release at the synaptic junction causes several detrimental effects associated with altered activity of synaptic proteins, transcription factors, Ca^{2+} homeostasis, and other molecules critical for neuronal plasticity. These detrimental effects further disrupt the normal homeostasis of neuronal cells and thereby causing synaptic loss. Moreover, the precise mechanistic role played by impaired NTs and neuromodulators (NMs) and altered redox signaling in synaptic dysfunction remains mysterious, and their possible interlink still needs to be investigated. Therefore, this review elucidates the intricate role played by both defective NTs/NMs and altered redox signaling in synaptopathy. Further, the involvement of numerous pharmacological approaches to compensate neurotransmission imbalance has also been discussed, which may be considered as a potential therapeutic approach in synaptopathy associated with AD.

Keywords: Amyloid-β, neurotransmitters/neuromodulators, redox signaling, synaptic dysfunction, tau, therapeutics

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INTRODUCTION

To maintain brain homeostasis, synapses and their associated neurotransmitters (NTs) play the role where synapses are specialized structures that form a network to transmit electrochemical signals

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Review

Re-expression of cell cycle markers in aged neurons and muscles: Whether cells should divide or die?

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ABSTRACT

Emerging evidence revealed that abrogated cell cycle entry into highly differentiated mature neurons and muscles is having detrimental consequences in response to cell cycle checkpoints disruption, altered signaling cascades, pathophysiological and external stimuli, for instance, Aβ, Parkin, p-tau, α-synuclein, impairment in TRK, Akt/GSK3β, MAPK/Hsp90, and oxidative stress. These factors, reinitiate undesired cell division by triggering new DNA synthesis, replication, and thus exquisitely forced mature cell to enter into a disturbed and vulnerable state that often leads to death as reported in many neuro- and myodegenerative disorders. A pertinent question arises how to reverse this unwanted pathophysiological phenomenon is attributed to the usage of cell cycle inhibitors to prevent the degradation of crucial cell cycle arresting proteins, cyclin inhibitors, chaperones and E3 ligases. Herein, we identified the major culprits behind the forceful cell cycle re-entry, elucidated the cyclin re-expression based on disturbed signaling mechanisms in neuromuscular degeneration together with plausible therapeutic strategies.

1. Introduction

The etiology of neuromuscular degeneration is characterized by the canonical deposition of non-functional/toxic proteins such as amyloid β, tau, parkin, α-synuclein, mutant huntingtin, and chronic inflammations in neuromuscular disorders (NMDs) including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Inclusion body myositis (IBM) and Poly myositis (PM). The deposition of pathological proteins or inclusions creates a physiological burden on cell that triggers multiple signaling cascades including mitogen-activated protein kinase (MAPK), c-Jun N-Terminal Kinases (JNK), Phosphatidylinositol 3-kinase/serine threonine kinase Akt/Glycogen synthase kinase 3 (PI3K/Akt/GSK3β), Notch and apoptotic signaling pathways. The consequent overexpression of cell cycle markers has been observed with the symptoms of cell cycle re-entry (CCR) [1]. Moreover, the co-localization of cell cycle markers with pathological proteins has reinforced the role of cell cycle machinery as a trigger to degeneration [2]. Further evidence suggests that re-expression of cell cycle markers occurs at prodromal stages before the appearance of pathological hallmarks in these neuromuscular disorders. A plethora of studies have demonstrated the involvement of oxidative stress, aging, mutation, mitochondrial dysfunction; ubiquitin proteasome system (UPS) shut down and loss of function of several protective proteins in the pathogenesis of these NMDs. For instance, prolonged activation or under the influence of acute insults such as DNA damage, oxidative stress, neurotrophic factor deprivation and altered microRNA (miR) function; cell cycle machinery turns detrimental and drives the cascade of DNA synthesis and consequent neuronal death culminating into neurodegeneration [3]. Interestingly, numerous studies have established cell cycle re-entry as a true phenomenon in neurodegeneration with actual DNA synthesis and cannot be viewed as an epiphenomenon of other processes. Moreover, cell cycle markers' re-expression in terminally differentiated neurons is also reported to fulfill the physiological demands pertaining to synaptic plasticity, neuronal migration and maturation [4]. Importantly, current studies advocate that aberrant cell cycle re-entry is not a consequence, but rather a cause of neuromuscular degeneration, depicting the importance of targeting cell cycle re-entry as a therapeutic window for treating neuromuscular disorders. These days several compounds/biomolecules have been implicated for targeting cell cycle re-entry in neurons and muscles. Moreover, potential role of ubiquitin E3 ligases and heat shock proteins have also been demonstrated to revert the damaging effect caused by cell cycle re-entry in neuromuscular disorders.

2. Relevance of cell cycle markers (cyclins, CDKs, CDKIs) in maintaining cell cycle homeostasis

The eukaryotic cell cycle is orchestrated machinery that accomplishes fundamental roles in cell replenishment such as DNA replication,

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Linking mitochondrial dysfunction, metabolic syndrome and stress signaling in Neurodegeneration☆

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ABSTRACT

Mounting evidence suggests a link between metabolic syndrome (MetS) such as diabetes, obesity, non-alcoholic fatty liver disease in the progression of Alzheimer's disease (AD), Parkinson's disease (PD) and other neurodegenerative diseases (NDDs). For instance, accumulated Aβ oligomer is enhancing neuronal Ca^{2+} release and neural NO where increased NO level in the brain through post translational modification is modulating the level of insulin production. It has been further confirmed that irrespective of origin; brain insulin resistance triggers a cascade of the neurodegeneration phenomenon which can be aggravated by free reactive oxygen species burden, ER stress, metabolic dysfunction, neuorinflammation, reduced cell survival and altered lipid metabolism. Moreover, several studies confirmed that MetS and diabetic sharing common mechanisms in the progression of AD and NDDs where mitochondrial dynamics playing a critical role. Any mutation in mitochondrial DNA, exposure of environmental toxin, high-calorie intake, homeostasis imbalance, glucolipotoxicity is causative factors for mitochondrial dysfunction. These cumulative pleiotropic burdens in mitochondria leads to insulin resistance, increased ROS production; enhanced stress-related enzymes that is directly linked MetS and diabetes in neurodegeneration. Since, the linkup mechanism between mitochondrial dysfunction and disease phenomenon of both MetS and NDDs is quite intriguing, therefore, it is pertinent for the researchers to identify and implement the therapeutic interventions for targeting MetS and NDDs. Herein, we elucidated the pertinent role of MetS induced mitochondrial dysfunction in neurons and their consequences in NDDs. Further, therapeutic potential of well-known biomolecules and chaperones to target altered mitochondria has been comprehensively documented. This article is part of a Special Issue entitled: Oxidative Stress and Mitochondrial Quality in Diabetes/Obesity and Critical Illness Spectrum of Diseases - edited by P. Hemachandra Reddy.

1. Introduction

For the maintenance of energy metabolism and cellular homeostasis mitochondria is an important organelle which is also known as the power house of cells and predominantly required for determining many cellular functions ranging from metabolic to catabolic activities. Mitochondria performs numerous crucial functions within the cell, which include cellular ATP production, Ca^{2+} buffering, regulation of apoptotic process and involvement in the synthesis of key metabolites. Nevertheless, it also acts as a primary source of endogenous reactive oxygen species (ROS) under oxidative stress. Additionally, mitochondria provides most of the ATP for the metabolic and cellular reaction within the cell, which is mainly coupled with electron transport system (ETS) [1,2]. However, research in the past few decades has recognized various factors, such as mutations in mitochondrial DNA and environmental toxins causing homeostatic imbalances, consequently leading to the damage of normal mitochondrial dynamics. Such alterations include altered mitophagy, decelerated ATP production, disturbed Ca^{2+} homeostasis, reduced mitochondrial membrane potential and compromised mitochondrial respiration [3]. Since, the potential mechanistic role played by altered mitochondria and their associated risk factors in MetS and NDDs remain unsettled, and their possible interlinking is still needed to be investigated. This review extensively covers the involvement of mitochondrial dysfunction in both MetS and neuronal dysfunction. Further, implementation of several biomolecules and chaperones for targeting MetS and NDDs induced by mitochondrial dysfunction has also been elaborated.

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Ion Channels in Neurological Disorders

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Abstract

The convergent endeavors of the neuroscientist to establish a link between clinical neurology, genetics, loss of function of an important protein, and channelopathies behind neurological disorders are quite intriguing. Growing evidence reveals the impact of ion channels dysfunctioning in neurodegenerative disorders (NDDs). Many neurological/neuromuscular disorders, viz, Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis, and age-related disorders are caused due to altered function or mutation in ion channels. To maintain cell homeostasis, ion channels are playing a crucial role which is a large transmembrane protein. Further, these channels are important as it determines the membrane potential and playing

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Review Article

Molecular Chaperones and Ubiquitin Proteasome System in Tumor Biogenesis: An Overview

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Abstract

The hallmark feature in cancer is uncontrolled cell-division and altered protein expression. Currently, cancer is one of the most detrimental diseases encountered by a large population across the globe. However, an absolute treatment strategy has still not been achieved by the researchers. Further, molecular mechanism and therapeutic to combat this lethal disease is a baffling issue. Molecular chaperones and ubiquitin proteasome system is mainly responsible for the maintenance of protein homeostasis and thus playing a crucial role in the cancer pathophysiology. Molecular chaperones are a superfamily of proteins which expressions are triggered under physiological, pharmacological and environmental insults and playing a protective role for cell survival. However, beyond a threshold of protection, molecular chaperones are unable to provide proper shape of non functional proteins that accumulate unwanted protein the cellular milieu. In order to get rid off these accumulated proteins ubiquitin proteasome system comes into action where an E3 ligase, specific enzymes of ubiquitination system, play a decisive role in the turnover of many essential regulatory proteins involved in cancer. It also mediates numerous functions, for instance, cell death, cell growth and DNA repair. Since, both molecular chaperones and E3 ligases have been involved in the progression of cancers it is necessary to understand and implement the role of these two molecules to use as diagnostic markers to treat cancer. Herein, we have comprehensively discussed the functional role of molecular chaperones, their differential protein expressions and a possible correction mechanism in cancer. Furthermore, comprehensive information has been documented regarding E3 ligases and their associated role in cancer that may be used as potential diagnostic biomarker for the treatment of various cancers.

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Introduction

The progression of cancer is so silent that it is hard to diagnose at the earlier stages, and it is an equally challenging task for the clinicians to treat this disease at early stage due to many unknown mechanisms with sites and tissues specificity issues. Cancer cells are immensely proliferatives, incursive, and metastatic in nature. The pathological hallmark in cancer is uncontrolled cellular proliferation and altered protein expression where hindrance in chaperoning machinery, and UPS is one of the major reasons. Molecular chaperones and ubiquitin E3 ligases are ubiquitous class of proteins that play an essential role in the conformational quality control of the proteins through interacting with various misfolded proteins, stabilizing and remodeling a wide range of non-native polypeptides [1,2]. Even though constitutively expressed under balanced growth conditions, many chaperones are upregulated upon exposure of heat shock or other insults that constitutively increases cellular protein level and for that reason, it is also known as stress or Heat Shock Proteins (HSPs) [3]. Molecular chaperones act together on unfolded or partially folded protein subunits, for instance, nascent chains emerging from the ribosome and with extended chains being translocated across sub-cellular membranes [4]. In addition, some chaperones are non-specific, and they easily interact with a broad range of protein subunits, while others are restricted to specific targets. They often require ATP binding/hydrolysis for their proper functions [4]. Moreover, they also stabilize the non-native conformation for correct folding of protein subunits [5] and not associate with native proteins nor do they form part of the final folded structures. HSPs were first identified as a group of proteins, which are brought about by heat shock and other kind of sources, including physical and chemical stressors in a broad range of species [6]. The HSPs have been afterwards described as molecular chaperones, which share common property of altering the structures and interactions of other proteins [7].

Importantly, molecular chaperone directs that the HSPs frequently interact in a stoichiometric manner with their substrates and also require high intracellular levels of proteins [6]. This is the reason why HSP28, HSP40, HSP70 and HSP110 genes have developed a highly effective mechanism for their synthesis during stress with transcriptional activation, efficient messenger RNA (mRNA) stabilization and their preferential translation [8] and boosts to become dominantly expressed proteins under such stress scenario [9]. Heat shock factor (hsf) family is the main regulatory machineries for HSP gene transcription, which ensure proper transcriptional activation and equally steep switch-off mechanism after recovery [10]. The hsf gene family comprises heat shock transcription factor 1 (hsf1), acts as central co-ordinator of heat shock stress response. Besides this, heat shock transcription factor 2 (hsf2) and heat shock transcription factor 4 (hsf4) are also involved in stress response but less active with respect to hsf1 [11]. HSP transcription needs activated hsf1, which is itself over-expressed in cancer and play a crucial role in invasion and metastasis [12]. Further, the molecular

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Review Article

p38 MAPK and PI3K/AKT Signalling Cascades in Parkinson's Disease

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Parkinson's disease (PD) is a chronic neurodegenerative condition which has the second largest incidence rate among all other neurodegenerative disorders barring Alzheimer's disease (AD). Currently there is no cure and researchers continue to probe the therapeutic prospect in cell cultures and animal models of PD. Out of the several factors contributing to PD prognosis, the role of p38 MAPK (Mitogen activated protein-kinase) and PI3K/AKT signalling module in PD brains is crucial because the impaired balance between the pro- apoptotic and anti-apoptotic pathways trigger unwanted phenotypes such as microglia activation, neuroinflammation, oxidative stress and apoptosis. These factors continue challenging the brain homeostasis in initial stages thereby essentially assisting the dopaminergic (DA) neurons towards progressive degeneration in PD. Neurotherapeutics against PD shall then be targeted against the misregulated accomplices of the p38 and PI3K/AKT cascades. In this review, we have outlined many such established mechanisms involving the p38 MAPK and PI3K/AKT pathways which can offer therapeutic windows for the rectification of aberrant DA neuronal dynamics in PD brains.

Key words: Parkinson's disease (PD), p38MAPK, PI3K/AKT, neuroinflammation, oxidative stress (OS), apoptosis, neurotherapeutics

Neurodegenerative disorders (NDs) continue to traumatize an aging proportion of the human population especially in the industrialized world. Aging has long been recognized as a compound process of damage accretion that ultimately leads to noticeable disruption of multiple cellular and molecular proceedings, which ultimately are translated into various chronic ailments such as Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD), atherosclerosis and many more (1-3). Although, several NDs have a pharmacological treatment, which as in the case of AD, PD, epilepsy and MS slow down the course of the disease, and are restricted to damage limitation,

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Review

Tau Phosphorylation, Molecular Chaperones, and Ubiquitin E3 Ligase: Clinical Relevance in Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) is characterized by dementia, cognitive disabilities, and tauopathy. Tau is a microtubule associated protein that helps maintain the neuronal network. While phosphorylation of tau protein causes disruption of the microtubular network, dephosphorylation allows reconstitution of the microtubule network. Several kinases, e.g., MARK, MAPK, and protein kinase C, are known to hyperphosphorylate tau, leading to disruption of the microtubular network and formation of neurofibrillary tangles (NFTs), which are further glycosylated, glycated, and have lipid peroxide adducts that impair the neuronal transport system and affect memory formation and retention. Moreover, intracerebral administration of amyloid-β oligomers causes hyperphosphorylation of tau, but whether it is involved in the formation of NFTs is still unclear. Further, amyloid burden activates AMP-activated protein kinase that increases phosphorylation of tau at position Ser262/Ser356 and Ser396. Several phosphatases are present at low levels in AD brains indicating that their down regulation results in abnormal hyperphosphorylation of tau. However, evidence strengthens a possible link between tau phosphorylation and molecular chaperone mediated tau metabolism for the clearance of toxic tau accumulation and has a crucial role in tauopathy. Furthermore, accumulation of phosphorylated tau protein and the possibility of removing the toxic phosphorylated tau protein from the milieu indicates that the chaperone interacts with phosphorylated tau and promotes its degradation. For instance, Hsp90 and cdc37 regulate tau stability and phosphorylation dynamics whereas Hsp27 is able to modulate neuronal plasticity, while 14-3-3 is involved in the interaction of tau with small HSPs. Hsp70 ATPase acts as a modulator in AD therapeutics while Hsc70 rapidly engages tau after microtubular destabilization. Herein, we highlight the various causes of tauopathy and HSP-E3 ligase mediated therapeutics in AD.

Keywords: Heat shock proteins, tau phosphorylation, tauopathy, ubiquitin E3 ligase

INTRODUCTION

The cytoskeletal structure of neurons plays an important role in maintaining cell structure, shape, and function. The cytoskeleton is primarily composed of microtubules. These microtubules are maintained and stabilized by a major type of microtubule associated

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RESEARCH

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Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules

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Abstract

Background: Angiogenesis is a hallmark feature in the initiation, progression and growth of tumour. There are various factors for promotion of angiogenesis on one hand and on the other hand, biomolecules have been reported to inhibit cancer through anti-angiogenesis mechanism. Biomolecules, for instance, luteolin, lectin and lupeol are known to suppress cancer. This study aims to compare and evaluate the biomolecule(s) like luteolin, lupeol and lectin on CAM assay and HT-29 cell culture to understand the efficacy of these drugs.

Method: The biomolecules have been administered on CAM assay, HT-29 cell culture, cell migration assay. Furthermore, bioinformatics analysis of the identified targets of these biomolecules have been performed.

Result: Luteolin has been found to be better in inhibiting angiogenesis on CAM assay in comparison to lupeol and lectin. In line with this study when biomolecules was administered on cell migration assay via scratch assay method. We provided evidence that Luteolin was again found to be better in inhibiting HT-29 cell migration. In order to identify the target sites of luteolin for inhibition, we used software analysis for identifying the best molecular targets of luteolin. Using software analysis best target protein molecule of these biomolecules have been identified. VEGF was found to be one of the target of luteolin. Studies have found several critical point mutation in VEGF A, B and C. Hence docking analysis of all biomolecules with VEGFR have been performed. Multiple allignment result have shown that the receptors are conserved at the docking site.

Conclusion: Therefore, it can be concluded that luteolin is not only comparatively better in inhibiting blood vessel in CAM assay, HT-29 cell proliferation and cell migration assay rather the domain of VEGFR is conserved to be targeted by luteolin, lupeol and lectin.

Keywords: CAM assay, Flavonoids, HT-29 cell, Anti-angiogenesis, Luteolin, Lupeol, Lectin

Background

Angiogenesis process is regulated by several factors that have a critical role in governing the initiation and progression of tumour. Angiogenic factors such as bFGF, HGF, VEGF, hyluronatelyase, collagenase, MMP supports the formation of new blood vessels. In addition, cell cycle markers, for instance, cyclin A2, Cyclin Dependent Kinase-2, 6 and MAPK1, 14, 10 promote the tumour progression whereas caspase 3 inhibits the tumour progression. Mounting evidence is suggesting the critical role of cyclin inhibitors, and inducers of apoptotic markers in cancer therapy. Furthermore, several biomolecules elicit the anti-cancerous property such as, luteolin, lectin and lupeol but comparative studies in terms of anti-angiogenic activity remain unsettled.

Luteolin is a flavonoid; lupeol is a triterpene and lectin is a protein possessing carbohydrate. Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects, and UV

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Chapter 7
Epigenetics and Angiogenesis in Cancer

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7.1 Introduction

Conrad Waddington was the first to coin the term "epigenetics" in 1939, and defined as "any heritable changes in a cellular phenotype without altering the DNA sequence" [1]. Epigenetic is the phenomenon which describes the event eventually involving chromatin mediated regulatory process of DNA-template. Moreover, highly regulated machineries are involved in the process of DNA and histones' modification and their removal by chromatin-modifying enzymes while, DNA modifications are of four different types [2, 3], histone modifications have 16 classes [4]. These modifications can alter non covalent interactions within and between nucleosomes and thus lead to modify the chromatin structure. These altered chromatin structures act as docking sites for particular proteins with unique domains that specifically identify these modifications.

Epigenetic modification plays an integral role in the regulation of all DNA-based processes, including DNA replication, transcription, and DNA repair. Furthermore, altered genome or irregular expression patterns of chromatin regulators may trigger different tumor cells to transform into malignant cells. Interestingly, epigenetics

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